American Journal of Veterinary Research

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The Transformation of the Lining of the Pulmonary Alveoli with Special Reference to Adenomatosis in the Lungs (Jagziekte*) of Sheep

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Onderstepoort, South Africa

DIFFERENCES of opinion exist regarding the type and function of the cells lining the alveoli of the lung. Some believe that there is a true alveolar epithelium, although it is difficult to demonstrate in the normal lung.

According to Jordan,[17] the pulmonary alveoli with extremely thin walls consist of a capillary network, a delicate fibroblastic reticulum, and a lining of epithelium which is continuous with that of the respiratory bronchioles; the lining epithelium consists of flattened cells and broad protoplasmic non-nucleated plates. The normal respiratory epithelium does not become phagocytic. Bell[2] believes that the alveolar walls of the postnatal lung are largely bare of epithelium, but that epithelial cells may occasionally be found; and that, although the lining epithelial cells are inconspicous in the normal lung, there are several diseases in which the alveolar epithelium may undergo hyperplasia and form a continuous epithelial layer. Cowdry[7] maintains that a proper concept of the nature of the alveolar walls is difficult to convey, but that there is a possibility that scattered epithelial cells normally occur in the alveolar lining which, though disfigured by flattening, are capable of making their true nature known by their behavior.

Others hold that there is no true epithelium on the alveolar walls, but only mesenchymal cells which occur in sheets. According to Anderson,[1] the walls of the alveolar sacs and alveoli consist of dense nets of anastomosing capillaries supported by abundant interlacing reticular fibers; however, in the interstices of the capillary nets, numerous histiocytes in the "resting stage" lie close against the surfaces of the capillaries and, when stimulated, become alveolar macrophages.

Watts and MacDonald[31] state that under ordinary circumstances the alveoli of the adult do not have a visible lining of cells, but a discernible lining may be associated with many pathological conditions; that these cells are derived first from bronchiolar epithelium, and second from papillary cells of the alveolar wall; that the appearance of the cells of bronchiolar origin differs markedly from that of the cells of alveolar origin; and that the cells of alveolar origin are frequently phagocytic, even after they have assumed a typical epithelial-like structure and arrangement.

Many pathologists, according to Geever and Neubuerger,[14] believe that no definite alveolar lining is evident in the lungs of healthy cattle, sheep, and hogs but that, under pathological conditions, there may be all stages from a simple swelling of the septal cells to the formation of a continuous lining and, finally, to actual neoplastic growth. They discuss the origin and development of the epithelial-like alveolar lining cells and their probable mesenchymal character.

Low[18] maintains that the differences of opinion regarding the nature of the pulmonary epithelium in mammals might be traceable to technical limitations largely unavoidable in light microscopy; that the electron microscope has revealed that there is in mammals, including man, a nucleated epithelium lining the walls of the pulmonary alveoli; and that it is a true respiratory epithelium.

Controversy has arisen as to whether pulmonary-cell neoplasms in animals and man originate from bronchiolar epithelium or alveolar cells, and whether the latter cells are epithelium or mesothelium. Cowdry[6] stated that, in adenomatosis of the lungs of sheep (jagziekte in South Africa), epithelial proliferations of alveolar origin arise only in lungs modified by a thickened interalveolar tissue. There is a metamorphosis of the epithelium

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The author is indebted to Professor H. L. de Waal, Pretoria University, South Africa, for his guidance and assistance with the chemical preparations used in these experiments, and also wishes to thank Professor J. C. Thompson and members of the staff of the Pathological Institute, University of Cape Town, South Africa, for their advice and help.

* The Afrikaans spelling is "Jaagsiekte."

into a single layer of cubical cells or, more frequently, of columnar cells, which may assume a papillomatous arrangement.

De Kock [8] described the histology of lung adenomata in the lungs of sheep affected with jagziekte in South Africa. Various stages of cell proliferation could be followed in the air saes and, sometimes, in the lumina of bronchioli, from the formation of low cuboidal cells on the walls of the alveoli to multiple adenomata "bulging" into the alveolar spaces, alveolar duets, and lumina of the bronchioli. There was no evidence of an infiltrative growth, nor was any metastasis observed. Dungal,[13] in describing a disease in sheep in Iceland, apparently similar to jagziekte in South Africa, indicated that some of the lesions displayed a distinct adenomatous character; some could be traced to the bronchial epithelium, and others to the alveolar lining.

It is believed that the tumors in the lungs of certain strains of mice are true neoplasms. They appear to be of alveolar and bronchiolar origin. In the early stages, they show a simple thickening of the alveolar epithelium, with an approach to a cuboidal character. As the process continues, papillary outgrowths form in the alveolar spaces and, finally, columnar-cell papillary growths may be formed. Campbell [5] indicates that the lung tumors in mice usually appear as simple adenomata from the epithelium of the alveoli, although adenomatous cells may also have extended from the bronchial epithelium.

Neubuerger and Geever [24] list alveolar cell tumors in man and indicate that most of the evidence points to the existence of a specific tumor arising from the cells lining the alveolar walls. They seem to prefer the term "alveolar-cell" tumors because of the uncertainty as to their histogenesis. The trend of opinion, according to them, favors the idea that the alveoli of the adult lung are not lined by epithelium. This type of tumor probably does not form more than 5 per cent of the pulmonary neoplasms in man. Simon [27] states that the interesting problem raised by pulmonary adenomatosis is the question regarding the presence and nature of cells lining the alveoli. In pulmonary adenoma, these cells are of an epithelial nature, but it is difficult to say whether these proliferating cells spring from pre-existing epithelial cells or represent a metaplasia from an indifferent cell type. Swan [30] submits a table of cases of pulmonary adenomatoses (without metastasis) in man. In the five cases, tall cubical columnar or cylindrical cells lined the pulmonary alveoli, forming a single layer. In some instances, papillary projections of tumor cells jutted into the alveolar spaces, and one case closely resembled jagziekte lesions in sheep.

The incidence and cause of lung tumors in sheep have been studied in various countries. In South Africa, the morbidity from jagziekte in Merinos is extremely low, and only isolated cases occur in certain areas. It does not spread like an infectious disease, and contact experiments at Onderstepoort have been inconclusive.

Dungal and Gislasen [11] stated that jagziekte in sheep was probably introduced into Iceland by a Karakul ram from Germany in 1933 and, by 1935, approximately a third of the sheep on the island were affected. Transmission by contact was easily brought about by housing sheep together during the winter months in small, badly lighted houses with no special provision for ventilation. There was also a suspicion that certain breeds of sheep were particularly susceptible to jagziekte. The results of their transmission experiments to identify an "infection entity" were inconclusive.

McFadyean,[22] in referring to his previous publication (1920[21]), reported that jagziekte was in England at that time, but that it is more reasonable to assume that the lesions were not true neoplasms but inflammatory new growths. Taylor [30] indicates that the lesions in the lungs of British sheep, referred to by McFadyean in 1889, 1894, and again in 1920, were not those of a lungworm infection but were lesions of a disease similar to, if not identical with, jagziekte in South Africa. From the publications of Dungal, it would seem that, in the lungs of sheep in Iceland, a Muellerius infection may occur simultaneously with jagziekte lesions. Blakemore and Bosworth [3] contend that, in their cases of jagziekte in England, the general appearance of the lesions in the lungs resembles a neoplastic condition, in which no metastasis was found in the thoracic lymph nodes. They support the view that typical proliferative changes of the epithelium of jagziekte could occur in the absence of parasites in the lung. Bull [4] reports that, although Muellerius infection is fairly common in Australia, there have never been cases of jagziekte to complicate the problem.

According to Pallaska,[25] Eber referred to primary adenomatosis of the lungs of sheep in Germany as early as 1891, but Nieberle was doubtful about the conclusions drawn by Eber. Nieberle believed that, in strongylosis of the lungs of sheep, adematous-like proliferations were not infrequent. Pallaska, however, believed that such a proliferation of epithelial cells in a fibrillar substrate was not a metaplasia, hypertrophy, or hyperplasia, but an attempt at regeneration. An autonomous neoplastic growth was not observed in verminosis of the lung, but the changes were more probably associated with a chronic inflammatory process.

The etiology of lung tumors of mice has been widely discussed. Murphy and Sturm [23] indicate that the external application of tar results in a high incidence of lung tumors in mice (60–80%). The incidence for corresponding age periods and stock in which spontaneous lung tumors had been frequent was never more than 5 per cent. According to Lynch,[19] the "neoplasm age" or "tumor rate" in mice may have been influenced by tarring, but Lynch [20] stated that there was evidence in favor of the theory that susceptibility to spontaneous lung tumors in mice was inherited.

Campbell [5] suggests that lung tumor susceptibility is inherited as a dominant character in mice. He concludes that some evidence has been obtained in experiments with mice that road dusts increase

the incidence of lung tumors, especially when tar is present. He accepts the irritant theory for cancer of the lung: that there may be several irritants coming from the external environment.

Shimkin [20] produced primary lung tumors in over 90 per cent of a strain of mice within four months after the intratracheal introduction of 1-2-5-6-dibenzanthracene or methylcholanthrene. Grady and Stewart [15] induced pulmonary neoplasms in strain A albino mice (with a normal spontaneous incidence of primary lung tumors), by the subcutaneous injection of dibenzanthracene or methylcholanthrene. Multiple tumors began to develop five weeks after the injection. The tumors were all of alveolar origin, and they began as a layer of cuboidal cells lining the alveolar wall. These cells morphologically resembled epithelium, unassociated with any recognizable inflammatory reaction.

Young [23] observed varied and extensive metaplasia of the serous membranes of the pulmonary pleura and hyperplasia of the pulmonary epithelium after the injection into the pleural sac of a mixture of Sudan III and sodium cholate in olive oil. In further experiments, he showed [23] that active proliferation of the epithelial cells lining the marginal alveoli can be produced in the rabbit by the intrapleural injection of an emulsion of liquid paraffin and bile salts, as well as with injections of chlorides of sodium, calcium, strontium, and aluminium. These results indicated to him that the changes brought about might be expressions of some general principle governing cellular activity.

According to Fried,[13] the lungs probably play a role in the metabolism of lipoids, and alkaloids and various toxic substances are oxidized by the pulmonary parenchyma. The pulmonary reaction to the intratracheal injection of an oil brings forth a histiocytic type of cell. The lung abounds in histiocytes and acts with amazing promptness.

De Kock [10] discussed the problem of phenol poisoning in sheep after dipping them in a proprietary dip. In experiments with this dip, avoiding any possible chance of ingestion of the dip by the sheep, absorption through the intact and uninjured skin apparently occurred. Marked signs of respiratory distress occurred within 24 hours and death within 48 hours. Apart from a general cyanosis and widespread vesicular and interstitial emphysema, the walls of the majority of alveoli showed a lining of cuboidal-like cells. In places, this lining was separated from the swollen and tortuous alveolar capillaries, probably as a result of an abnormal pressure by the accumulated air. These lining cells were regarded to be of the nature of epithelial cells and derived from the surface of the alveolar wall. After the intravenous injection of a mixture of tar, Simonds and Curtis [20] observed, in rabbits, circulatory disturbances, fibrous encapsulation of necrotic areas, and marked proliferation of the bronchiolar and alveolar epithelium about the encapsulated necrotic areas. They concluded that these proliferations were massive and atypical in many of the animals but apparently were not neoplastic, mainly because of their intimate and close association with granulation tissue, and the absence of any discernible metastasis.

In this brief review of the literature, it will be seen that no unanimity has been reached regarding the structure and function of the cells of the alveolar walls with the existing methods of staining and light microscopy. Some are of the opinion that there is a true epithelium lining the walls of the pulmonary alveoli, whereas others hold that these cells are mesenchymal in nature and that histiocytes in the resting stage lie close against the surface of the capillaries of the alveolar wall. The nature of these cells has been studied under many pathological conditions (e.g., in inflammation, neoplasms, etc.), and by the introduction of many irritants into the lungs.

Primary alveolar-cell adenomata have been described in the lungs of man, sheep, and mice. In mice and sheep, these tumors appear in the lumina of the alveoli and bronchioli and are not of an infiltrating nature. As far as it is known, no metastasis has been observed. The opinion is held that these neoplasms in the lungs of mice and sheep arise from the epithelium of alveolar and bronchiolar origin. The term "alveolar-cell adenoma" has been considered by some to be preferable for this type of tumor in the lungs of man, because of the uncertainty as to their histogenesis.

In certain strains of mice, the application of coal tar and certain carcinogenic hydrocarbons not only influenced the occurrence of lung tumors at a lower age level (4 months), but also induced a much higher incidence. There appears to be evidence in favor of the theory that susceptibility to spontaneous lung tumors in mice is inherited. It has been suggested that certain breeds of sheep are more susceptible to lung tumors.

From the available information, no conclusion has been reached regarding the cause of jagziekte in sheep. Apparently the disease can, under certain circumstances, be transmitted by close contact but, so far, the actual "inciting factor" has not been disclosed. Nematode infection of the lungs in sheep definitely can be ruled out as a direct cause. The epithelial-like proliferations in uncomplicated cases of verminous pneumonia are of the nature of a regenera-

tion, observed in certain forms of chronic inflammations of the lung.

In view of some of these problems, experiments were undertaken with respect to: (1) the etiology of jagziekte with special reference to *(a)* the possible existence of an inherited susceptibility, and *(b)* the presence of an "infection entity"; and (2) the reactions to irritants introduced into the lungs of sheep.

MATERIALS AND METHODS

It is difficult to diagnose jagziekte in its earliest stage. Whether the sheep used for experiments were free of lung lesions had to be judged on an incidence basis.

The natural incidence of jagziekte in sheep at the Onderstepoort Institute was based on a fairly large number of sheep (1,500–2,000) introduced annually for the production of various vaccines. Cases of jagziekte were detected only occasionally. However, the disease was not infrequent in sheep on a farm belonging to the School of Agriculture, at Tweespruit, Orange Free State. In 1928, among some 600 sheep there, more than 25 cases of jagziekte developed. This farm was a useful source of infected animals for experiments at Onderstepoort. Necropsies were performed on all sheep in the experiments, whether they died or were killed at the end of the experiment.

STUDIES ON AN INHERITED SUSCEPTIBILITY IN JAGZIEKTE

Experiment S3299.—Since April, 1929, 28 sheep, the progeny of affected rams and ewes from Tweespruit, were transferred to Onderstepoort and placed in close contact with jagziekte-affected sheep. Of the 28 Tweespruit sheep, 26 died of the disease, 20 after one to 30 days and 6 after two to three months of contact. Taking the usual course of this disease into consideration, there is no doubt that the majority were affected with jagziekte before they were sent to Onderstepoort. This confirmed the high incidence of this disease on the farm at Tweespruit.

Experiment S5063.—Since November, 1932, 27 sheep from areas regarded as free of the disease were placed in contact with known jagziekte-affected sheep for periods varying from six months to two years. Jagziekte lesions were found in only 1 animal, a clinically healthy sheep killed after 23 months.

Experiment S3767.—Further studies were made in April, 1930, on the incidence of jagziekte in Tweespruit ewes, and their progeny were mated with jagziekte-affected rams at Onderstepoort. Of 31 ewes, 12 had jagziekte lesions, mostly from six to 25 months after transfer to Onderstepoort. One ewe died on the third and 1 on the fortieth day; both were probably affected before transfer. Of the remaining 19 ewes, 10 apparently became affected at Onderstepoort. The results of this experiment again stress the high incidence of the disease in sheep bred from affected animals. Among the 29 first-generation ewes mated with affected rams at Onderstepoort, 10 (34%) had lesions in the lungs. Some of the surviving sheep were subsequently transferred to experiment S5246.

Experiment S3730.—Since May, 1929, ewes from an area believed to be free of jagziekte were mated with jagziekte-affected rams. Among 72 first-generation lambs, 25 (35%) had lesions at necropsy. It would, therefore, seem that the affected ram was able to influence the incidence of the disease. Some of the surviving sheep were subsequently transferred to experiment S5652.

When the results of these experiments are considered, it would appear that a susceptibility to jagziekte in sheep may be an inherited factor. The opinion is held in South Africa that the ram probably plays the major part with respect to an inherited disposition.

THE POSSIBLE EXISTENCE OF AN "INFECTION ENTITY" IN JAGZIEKTE

Experiment S4826.—An attempt was made, in October, 1932, to learn whether jagziekte could be transmitted to healthy sheep (from nonaffected areas) by the introduction of material from affected lungs. Emulsions were prepared from this lung material in normal saline solution, filtered through cotton wool, and 17 sheep were injected intrajugularly with 10 cc. of the emulsion. Some sheep were injected twice, at three-day intervals, while others were given three injections at weekly intervals; 11 of these sheep had been previously injected with coal tar (exper. S4739) before receiving the lung emulsions. An attempt was made to learn whether a lung of a sheep so injured would be more susceptible to a possible infection. As a result of the injections, 4 sheep died, while the remaining 13, killed from two months onward, showed no lung changes. Some of

the sheep had fibrosis in the lung, attributed to the previous coal tar injection.

Experiment S5398.—In June, 1934, emulsions of affected lungs were prepared as described. Aluminium hydrozide was added in a attempt to "separate out" a virus, if present. Intrapulmonary injections were given under anesthesia, in doses varying from 0.25 to 0.50 cc., to 14 sheep, some of which were also given 5 cc. intrajugularly. Three died within one to three days as a result of the injections, and the rest were killed 2.5 months postinjection. In none of these sheep was there any evidence of jagziekte at necropsy.

Experiment S7025.—In January, 1940, 2 sheep previously treated with benzpyrene (exper. 6506) were given intrapulmonary injections of lung emulsions from jagziekte-affected sheep, with negative results.

Experiment S6872.—In August, 1938, an experiment was begun to learn whether 5 sheep previously treated with carcinogenic hydrocarbons (exper. S6506, S6753) and 12 sheep (Texel breed) infected with lungworms *(Muellerius capillaris)* can become affected with jagziekte when placed in contact with affected animals. They were kept under observation for periods varying from several months to more than two years. Two died and the rest were killed. There was no evidence of jagziekte in the lungs of any of these animals.

One sheep (52375) previously given intrajugular and intrapulmonary injections of benzpyrene died 27 months after treatment. In the thoracic cavity, there was a neoplasm of the nature of an endothelioma or mesothelioma. At necropsy, the left lung was partly collapsed. The main lobe of the right lung was also partly collapsed and on its surface there were a number of small grayish transparent nodules, firm and almost fibrous in character. The cardiac and apical lobes were irregular in outline, soft, friable, and had a fleshy consistency. The spleen, liver, and kidneys showed evidence of atrophy. One section revealed fairly extensive fibrosis and lesions of necrosis in the pleura. Several sections of the lung showed areas of necrosis, but the greater part was comprised of cells of various shapes and sizes and, in places, closely packed together between much dilated capillaries. These cells varied from small to large, oval, cuboidal, or polyhedral cells with nuclei of various shapes and sizes, some centrally and others eccentrically situated. Some cells had more than one nucleus and some of the nuclei showed signs of necrosis.

REACTIONS TO IRRITANTS INTRODUCED INTO THE LUNGS OF SHEEP

To learn whether irritants introduced into the lungs of sheep could set up neoplasms and, if possible, to learn whether the lining cells of the walls of the alveoli are epithelial or mesothelial, the following experiments were carried out:

Experiment S4333.—In January, 1931, 4 sheep were dosed *(per os)* daily with 5 cc. of coal tar; some were given as much as 415 cc. in four months. None of the sheep showed any effects, and there was no evidence of neoplasia at necropsy.

Experiments S4739, 4900, and 4919.—During 1932, intrapulmonary injections of 0.2 to 0.5 cc. of coal tar were given to 37 sheep. One sheep died of shock and 8 others died in one to five days as a result of the injections. The main lesions observed in these animals were hemorrhages, areas of necrosis with coal tar droplets, and granulation tissue with numbers of leukocytes and macrophages. In some, there were also areas of pneumonia. Of particular interest in these cases was the proliferation of cells of the alveolar walls. These varied from scattered rounded and swollen cells to the partial lining of the proliferation of the alveolar epithelium was also seen in experiments referred to later. In some alveoli, these cells formed almost a complete lining for the alveolar walls.

Of the 37 sheep, 17 died or were killed at one month and at three months to one year and longer after treatment. In 2 of these, the lesions were confined to the visceral pleura, probably because the needle did not reach the lung tissue. In another sheep, the needle apparently penetrated the peritoneal cavity and caused a localized chronic peritonitis, with the presence of coal tar droplets. The lesions in the lungs varied from areas of granulation tissue to a fibrosis with coal tar droplets. These droplets varied in size, but were always circular and composed of a mass of small black pigment granules. In some sheep, the lungs also revealed lesions of necrosis, emphysema, and collapse. Fibrosis of the submucosa of the bronchi was also seen, with coal tar drop-

lets. Localized fibrosis of the pleura occurred in some of the cases. There was no evidence of tumor formation except in 1 sheep, in which jagziekte lesions were found at necropsy.

Of the 11 remaining sheep transferred to experiment S4826, 1 died of shock, and 4 died two to eight days after the injection. All had lesions of fibrosis as a result of previous treatment with coal tar. Some of the fibrotic nodules in the lung resembled the so-called paraffinoma.

THE APPLICATION OF CARCINOGENIC HYDROCARBONS

This was, apparently, the first time that sheep had been injected with carcinogenic hydrocarbons. The majority of animals used in these cancer studies have been mice, rats and, to a lesser extent, rabbits and guinea pigs. A number of important factors with regard to sheep had to be considered before these injections were undertaken. It was believed that the time from the first application of these preparations until the first signs were produced could be shortened if the application were repeated at short intervals.

The average age of a Merino sheep is approximately ten times that of rats, and 20 times that of mice. When the average weight is considered (mice, 30–50 Gm.; rats, approx. 300 Gm.; and a yearling sheep, 40 kg.), a sheep would theoretically require about 120 times as much hydrocarbons as a rat, and about 800 times as much as a mouse in order to achieve similar results.

Chemically pure synthetic 3-4-benzpyrene and methylcholanthrene and 1-2-5-6-dibenzanthracene were separately dissolved in sesame oil or in Merck's prepared lard. In each case, the concentration was a 2.5 per cent solution, and 1 cc. would, therefore, contain approximately 25.0 mg. of these hydrocarbons. After preliminary experiments with the injection of lard or sesame oil in control sheep (exper. S6754), the following method of treatment was adopted: On June 16, 1939, sheep were injected either intrajugularly, intratracheally, intrapulmonarily, or intramuscularly with 0.5 cc. of a 2.5 per cent solution of benzpyrene or methylcholanthrene in sesame oil or lard at weekly, two-week, or monthly intervals. The doses, especially those introduced into the lungs of sheep, were gauged by signs of shock or of poisoning.

Experiment S5246.—In December, 1933, the effects of weekly, two-week, or monthly intrapulmonary or intratracheal injections of dibenzanthracene were studied in *(a)* 15 sheep from a jagziekte-free source, and *(b)* 7 sheep, the progeny of ewes from a farm where this disease had occurred (Tweespruit).

Of the 15 sheep, 1 died on the day of injection and had jagziekte lesions in the lung. The others died or were killed at intervals of three weeks, 1.5 months, and 1.5 years, up to 2.5 years. There were signs of emphysema, collapse, hemorrhage, pneumonia, and fibrosis of the lung, but no evidence of neoplasms.

Of the 7 sheep, 1 died on the second day after the injection and had jagziekte lesions in the lungs. The others died or were killed from three months to more than two years postinjection, and had lesions as described for the first group. The 2 sheep in this experiment that died of jagziekte were undoubtedly affected before the injection.

Experiment S5652.—Beginning on April 16, 1935, the effects of intrapulmonary and intramuscular injections of dibenzanthracene in sheep from an area free of jagziekte were studied. Nine sheep were injected on alternate weeks with 0.2 to 0.6 cc. of dibenzanthracene solution intrapulmonarily and 2 cc. intramuscularly. One sheep died within 29 days, 1 was killed *in extremis* within 33 days, and 1 died 4.5 months after the beginning of treatment. Apart from congestion of the lungs, pneumonia, and fibrosis, proliferation of the cells of the alveolar walls of the same nature as that described for the preceding experiments was observed. The 6 remaining sheep were given approximately 28 intrapulmonary and 27 intramuscular injections between April 16, 1935, and May 2, 1936, when they were killed for necropsy. Apart from hemorrhage, emphysema, patches of bronchopneumonia, and well-marked fibrosis, there was no evidence of neoplasms in the lungs or pleura. In 1 of these sheep (32967), the muscles of the gluteal region at the site of injection had fibromatoid lesions. None of the sheep in this experiment had lesions of adenomatosis of the lung (jagziekte).

Experiment S6506.—On Aug. 9, 1939, 15 sheep were given either intrapulmonary

or both intrajugular and intrapulmonary injections of 0.5 cc. of a 2.5 per cent solution of 3.4 per cent benzpyrene at weekly intervals. Three sheep died from two to 4.5 months after the injections and, at necropsy, showed hemorrhages, necrosis, and fibrosis in the lungs. The remaining 12 sheep were given 18 to 80 cc. at weekly intervals. One sheep died after 11.5 months; the rest were subsequently killed for necropsy. The main lesion observed in the lungs was a fibrosis.

Experiment S6753.—On Aug. 28, 1939, 6 sheep were injected intrapulmonarily and intrajugularly either at weekly intervals or on alternate weeks with 0.5 cc. of a 2.5 per cent solution of benzpyrene. Three of these sheep died within three days, 1 within three weeks, and 1 in six months after the beginning of treatment. In some of these 5 sheep there were proliferations of cells of the alveolar walls. The remaining sheep (52388) had neoplasms in the thoracic cavity that were of the nature of an endothelioma or mesothelioma, with metastasis in a lymph node. The visceral pleura was markedly thickened and firmly adherent to the costal and diaphragmatic parietal pleura. There was partial collapse of the left and complete collapse of the right lung. Multiple tumors were present on the pleura and extended into the pericardial sac. In the pleural sections, there was a single tract of cells around the capillaries. These cells were cuboidocolumnar in appearance, and were vertical to the long axis of the lumen of the capillary blood vessels. In places, the lumen of the capillary had a homogenous pink appearance. These cellular structures, which radiated around the pink substance, had infiltrated into one of the lymph nodes, causing marked disintegration of the lymphoid tissue. In sections of the lung, there was evidence of collapse, emphysema, necrosis, and hemorrhage. In one section of the lung, there was a fair amount of granulation tissue, with active proliferation of the epithelial cells of the vascular capillaries and of the fibroblasts.

Five sheep were injected intrapulmonarily and intrajugularly either at weekly intervals or on alternate weeks with 0.5 cc. of a 2.5 per cent solution of methylcholanthrene. One sheep died on the following day, 1 in three weeks, and 1 in 7.5 months. The remaining 2 were killed several years afterward. The 1 sheep that died shortly after the injection had congestion and hemorrhage in the lungs, and marked necrosis was observed in the liver. The 1 sheep that died in three weeks had proliferation of the cells of the pulmonary alveolar walls, as well as fibrosis in the lungs.

Experiment S4439.—In view of Young's observations[37] of active proliferation of the epithelial cells of the pulmonary alveoli after the introduction of various salts into the lungs of rabbits, a similar study was undertaken in sheep. In June, 1931, 24 sheep were injected with 0.73 N strontium chloride solution intrathoracically, the doses varying from 10 to 50 cc. They were killed for necropsy three to seven days after the injection. The characteristic lesions in the lungs of these sheep were of the nature of an active proliferation of the cells of the alveolar walls, as observed in the other experiments.

RESULTS AND DISCUSSION

The high incidence of jagziekte in sheep bred on a farm in South Africa was confirmed in experiments carried out at Onderstepoort. However, only 1 case of the disease occurred in a number of sheep (regarded as free of this disease) when placed in close contact with affected sheep at Onderstepoort. When ewes from the jagziekte-free areas were mated with rams affected with the disease, the incidence of the disease in the progeny of these ewes was more than 34 per cent. The consensus of farmers in South Africa and in Iceland was that the affected sire was able to influence the suceptibility to this disease. From the results of the experiments, it would seem that breeding with affected ewes and rams demonstrated an inherited disposition in the progeny, which may explain why the contact experiments with sheep from a noninfected area were inconclusive.

The high incidence of jagziekte on the Tweespruit farm has been reduced to such an extent that the disease has not been encountered for a number of years. This is possibly a result of the policy of slaughtering all sheep at the earliest signs of any respiratory distress, and at the same time curtailing the driving and kraaling of sheep at night. It is possible that jagziekte at Tweespruit was gradually eliminated by: *(a)* the gradual removal of affected ewes and rams; *(b)* the preven-

tion of close contact of sheep in kraals at night; and *(c)* the possible "infection entity" in affected sheep gradually being eliminated.

In these experiments, it was not possible to isolate or identify the cause of lesions in the lungs. Not only were sheep from a clean area used, but lung emulsions from affected sheep were also introduced into the lungs of sheep previously treated with coal tar and carcinogenic hydrocarbons. Such treated sheep were also placed in contact with jagziekte-affected sheep for considerable periods with negative results.

The jagziekte lesions studied in these experiments resembled those previously described by De Kock.[9] The earliest lung changes seen at necropsy were small transparent nodules, usually irregularly distributed in the cranioventral aspect of the main lobe of the lung. At this stage, sheep show no signs of distress. As the disease progresses, reactions are manifested in the interalveolar capillaries, followed by bronchopneumonia, which becomes chronic and is associated with a fibrosis. As these changes progress, the sheep show various stages of respiratory distress which may last for several months before they die of a marked chronic bronchopneumonia and fibrosis of the lungs.

Microscopically, the earliest changes in the lungs can be detected in the vicinity of the scattered nodules. These are of the nature of a proliferation of cells of the walls of the alveoli, forming "budlike" swellings, and consist of cells resembling cuboidal epithelial cells. These "buds" continue to increase in size and eventually form a structure resembling a small papilloma which juts into the alveolar space. Cells in the single layer of epithelium gradually assume the size and shape of columnar cells. As this proliferation of epithelial cells progresses, the interalveolar capillaries become crowded with leukocytes, and an interalveolar reaction follows.

The classification of these tumors as papilliform cyst-adenomata seems to be justified. The belief that they are derived from the epithelium lining the walls of the alveoli and bronchioli also seems justified. However, the histiocytes and macrophages, which have such an important role in many diseases of the lungs, originate from the reticular tissue, as in all other organs.

Irritants, such as strontium chloride, coal tar, and certain carcinogenic hydrocarbons, were introduced into the lungs of sheep in single or repeated injections. Only a few animals died of shock or of other resulting complications. In sheep that died within a few days of treatment, there were characteristic lesions in the pulmonary alveoli: a proliferation along the alveolar walls which varied from scattered cuboidal cells to an almost complete lining of the wall of the alveolus with such cells. Some parts of the lung seemed adenomatous. There was no evidence of an intercellular stroma or of phagocytes with these epithelial-like cells. Similar changes have been described following the introduction of irritants into the lungs of rabbits, and the proliferating cells were also considered epithelial in character and to be derived from the alveolar wall. Similar lesions were also observed in the lungs of sheep that died as a result of phenol poisoning.

The prolonged treatment with carcinogenic hydrocarbons did not produce an adenomatosis in the lungs of sheep. Neoplasms of a different nature were, however, observed in the lungs and pleura of 2 sheep (52375 and 52388) after prolonged treatment with carcinogenic hydrocarbons. In view of the shape of the cells and their distribution in relation to the walls of the capillaries, a provisional diagnosis of endothelioma was made. To a certain extent, they resembled the mesothelioma described by Jackson,[16] who dealt with 1 equine and 2 bovine cases. From his own experience and from the information in the literature, there is no record of any other domesticated species being affected. He reports that much criticism has been leveled at the use of the term mesothelium, and comments on the controversial nature of the evidence regarding the histogenesis of these tumors.

There is a close resemblance between adenomatosis in the lungs of sheep and of that in the lungs of mice in the early stages, especially with respect to: *(a)* the primary origin of these tumors from the epithelium of the alveolar walls and bronchioli; *(b)* their morphology, spread, and distribution in the lungs; *(c)* their multiple appearance without any evidence of metastasis or an infiltrative method of growth; *(d)* the apparently inherited susceptibility to these neoplasms of both sheep and mice; and *(e)* the failure to isolate or identify the cause of adenomatosis in the lungs of either mice or sheep.

SUMMARY AND CONCLUSION

1) In the breeding experiments, it would appear that the progeny of rams and ewes affected with adenomatosis of the lungs (jagziekte) inherited a susceptibility for that disease.

2) On a farm which had a high incidence of jagziekte, the disease has not occurred for a number of years. This has probably been due to the policy of slaughtering all sheep showing the earliest evidence of this disease, and of preventing the kraaling of sheep. By killing affected sheep, the breeding of animals susceptible to adenomatosis was probably eliminated.

3) So far, it has not been possible to isolate or identify the cause of lung adenomatosis in sheep.

4) The tumors in jagziekte were classified as papilliform cyst-adenomata derived from the epithelium of the alveolar walls and bronchioles.

5) The close resemblance between adenomatosis in the lungs of sheep and that in the lungs of mice in the early stages was referred to.

6) The prolonged treatment of sheep with carcinogenic hydrocarbons introduced into their lungs failed to set up adenomatosis.

7) In the lungs and pleura of 2 sheep, subjected to prolonged treatment with carcinogenic hydrocarbons, neoplasms were found. A provisional diagnosis of endothelioma was made.

8) The introduction of certain irritants into the lungs of sheep revealed proliferations of the cells of the alveolar walls in those animals which died within a few days after treatment. The cells so formed were considered to be of an epithelial nature and derived from the walls of the pulmonary alveoli. These changes resembled lung lesions in similar experiments carried out in rabbits, and the lesions in the lungs of sheep that died of phenol poisoning. They were not like the changes observed in adenomatosis of the lungs of sheep.

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Pulmonary Adenomatosis in the Chinchilla

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PULMONARY ADENOMATOSIS (PA) has been reported in cows,[19,20,24] guinea pigs,[13] horses,[28] man,[1,2,12,14-17,21-23,25-27,30] mice,[29] and sheep.[3-6,8-11,18] This communication reports the disease in the domestic chinchilla *(Chinchilla laniger)*.

MATERIALS AND METHODS

A total of 206 chinchillas from ranches in Connecticut and neighboring states have been examined at this laboratory since 1942. Specimens were submitted in an effort to determine the cause of illness in a herd. The few living specimens received were killed with chloroform. At necropsy, representative tissues for histological study (brain, heart, lungs, stomach, liver, pancreas, duodenum, jejunum, colon, adrenal glands, kidney, and spleen) were fixed in formol-saline solution and embedded in paraffin. Harris' hematoxylin and eosin was used as a routine stain; special stains used were S Gram pyronin, Levaditi's silver technique, periodic acid Schiff (PAS), and Wolbach-Giemsa.

The hearts, livers, spleens, and lungs of the well-preserved specimens were examined by bacteriological methods, while the stomachs and livers were, in some instances, subjected to toxicological analysis.*

RESULTS

Pulmonary adenomatosis was seen in 19 of the 206 animals representing ten ranches (table 1). Eight of these 19 were submitted from one ranch over a period of six months; the owner considered the condition as enzootic. Although only 1 animal usually was presented, the owners invariably reported others similarly affected.

Clinical Signs.—The outstanding sign was dyspnea, often associated with lethargy. A progressive loss in condition occurred in animals which were ill for several weeks. Other signs, such as anorexia, enteritis, and wet chins and throats, were seen. It was difficult to establish the course of the illness, because the gradual and insidious onset obviated immediate detection. It was the consensus of the owners that the animals were ill from one to six weeks (table 1).

Gross Pathological Alterations.—The animals were generally considered to be in poor flesh, with a poor quality pelt. Nothing remarkable was observed in the lungs, except punctiform white areas. The enlarged "waterlogged" lung reported in other species[19,20,24] was not observed in chinchillas. The liver was normal in size, but sometimes had an orange hue. In noticeably debilitated specimens, this color change was pronounced and also occurred in the body fat.

Histological Alterations.—Pulmonary adenomatosis in the chinchilla expressed itself in a series of multicentric bilateral lesions, also observed in lungs of man[17] and sheep.[6,11] The affected areas usually were smaller than the field of the scanning lens of the microscope (x 32). The first detectable lesion was a patchy thickening of the alveolar walls, due to proliferation of microphages and limited infiltrations of lymphocytes. The effect was a multicentric interstitial pneumonia (fig. 1, 2, 3). The pathognomonic lesion was superimposed upon such areas. This lesion consisted of paving of the alveolar lining by a nonciliated, cuboidal-to-columnar epithelial cell which possessed pale, finely granular cytoplasm and a vesicular nucleus. Generally, this lining was uniform (almost precise), but it was not uncommon to see hyperplastic groups of cells forming papillae (fig. 4). The proliferating epithelial cell was larger than that of the normal respiratory bronchioles; such epithelium ceases at the beginning of the alveolar ducts (fig. 9). When stained with PAS, the cytoplasm of the proliferated alveolar cells revealed various degrees of staining intensity, suggesting some secretory function (fig. 4, 5).

In 6 animals, ill-defined foci of exudative pneumonia were seen. The exudate did not appear within the adenomatous areas, but did appear in the adjacent parenchyma. The pneumonia was not related to the duration of the illness (table 1).

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* The analyses were done by Dr. Harry Fisher, Connecticut Agricultural Experiment Station, New Haven.

Metastases to regional lymph nodes were not seen.

In all but one of the animals affected with PA, there were parenchymatous changes consisting of large vacuoles in the hepatic cells. Similar lesions were seen in animals poisoned by mercury and in animals in which anorexia was a prominent sign. The authors believed that these liver lesions, associated with PA, were secondary and the result of anorexia and subsequent starvation.

Other organs did not present pathological changes which could be associated with PA.

Bacteriological Examination.—From the ten lungs cultured, organisms were recovered only three times. A coliform organism, Staphylococcus sp., and *Alcaligenes faecalis* were recovered. Since most of the animals were submitted at a time when chinchillas were of considerable value, they had histories of intensive treatment with antibiotics. If an organism originally had been an etiological factor, its presence would have been no longer detectable. Virus isolation studies were carried out on 1 animal and are still in progress.

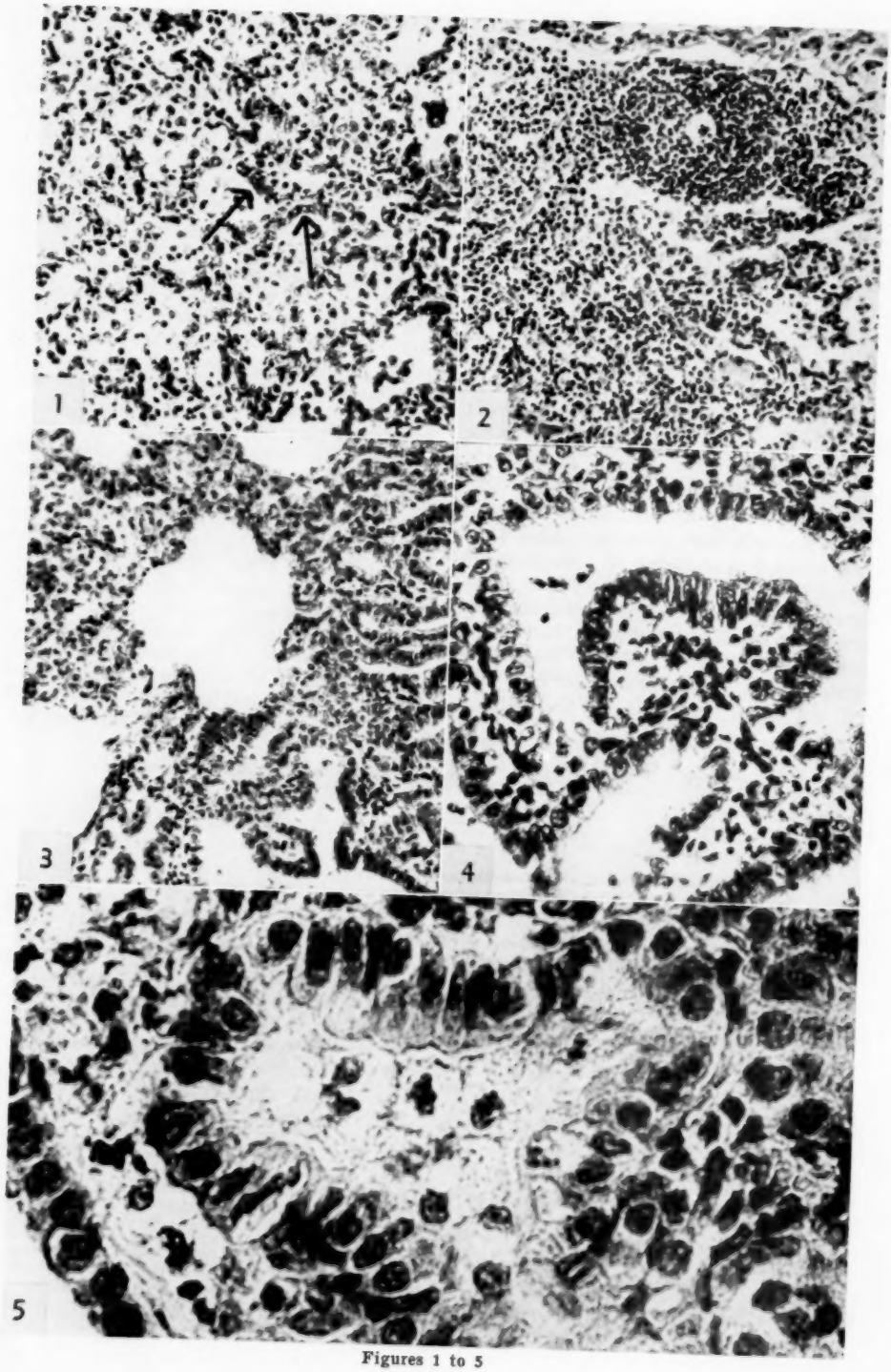
OTHER DISEASES

A variety of other disease conditions were observed in necropsies on 206 chinchillas. Aside from 39 specimens in which no diagnosis was made, the other large categories were ketosis and lipemia (32) and nephritis and nephrosis (24). These classifications were based on histological examinations. In the 32 animals classified as having ketosis and lipemia, the chief change consisted of vacuoles in the hepatic cells and the renal tubular epithelial cells, varying from slight to marked in extent. In the latter type, gross changes such as orange-colored liver and body fat were observed. Blood sugar levels of 2 such animals were just below 300 mg./100 ml., in comparison with normal values of 152 ± 24 for females and 132 ± 5 for males. The lesions in these animals were identical to those seen in animals with PA, in which the ketotic condition was considered due to starvation. In these animals, the fundamental cause apparently was entirely different, as such animals ate well.

In the 24 chinchillas classified as having nephritis and nephrosis, the lesions were not striking. The nephritic lesions consisted of slight focal infiltrations of small round cells in the cortical interstitium,

TABLE 1—Clinical Signs in Chinchillas with Pulmonary Adenomatosis

Chinchilla No.	Age (Years)	Sex	Clinical signs	Other remarks	Died (date)
1	1	F	Dyspnea, râles and rhinitis for 1 week.	.....	Jan., 1945
2	1	F	Dyspnea and facial alopecia for 6 weeks.	.....	July, 1950
3	6	M	Anorexia and lethargy for 3 weeks.	.....	Dec., 1950
4	2	...	Found dead without signs of illness.	.....	April, 1951
5	....	...	Dyspnea, anorexia and conjunctivitis for 12 weeks.	.....	Dec., 1951
6	½	...	Dyspnea and conjunctivitis for 12 weeks; appetite considered normal.	.....	Feb., 1952
7	¼	...	Owner believed animal "died of thimaine fits."	.....	Dec., 1952
8	1	F	Anorexia, lethargy, died suddenly; ill for 26 weeks.	.....	Dec., 1954
9	1	...	Conjunctivitis, slight colitis, poor coat, "not particularly fit."	.....	March, 1954
10	....	F	Photophobia, "nervous," anorexia, facial alopecia, loss of weight for 4 weeks.	.....	April, 1954
11	....	F	Conjunctivitis, poor coat, loss of weight for 8 weeks.	5 to 10 p.p.m. of mercury in liver.	April, 1954
12	1	M	No signs of illness.	.....	July, 1954
13	1	M	Loss of weight for undetected period.	.....	July, 1954
14	1	M	Loss of weight for undetected period.	5 p.p.m. of mercury in stomach and liver.	July, 1954
15	1	M	Conjunctivitis, facial alopecia, loss of weight for 8 weeks.	.....	July, 1954
16	1	M	Colitis for 2 weeks, followed by rectal prolapse and death.	.....	Sept., 1954
17	2	F	Dyspnea and sudden death, usually within one day; epizootic in character.	.....	Dec., 1955
18	1	F	Dyspnea; no other signs observed.	.....	Jan., 1956
19	1	F	Loss of weight, dyspnea, lethargy, ill for at least 10 days.	.....	Aug., 1957



Figures 1 to 5

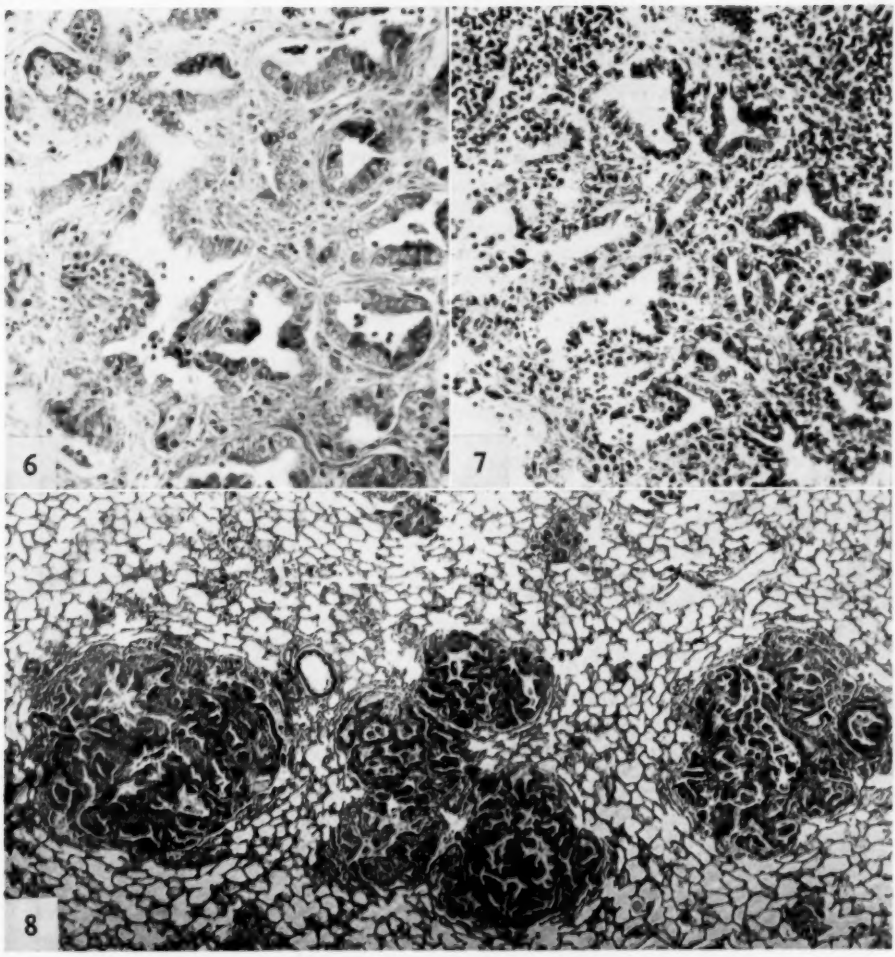


Fig. 6—Lung section from a Peruvian sheep. The deeper staining epithelial cells are believed to indicate secretory activity. Periodic acid Schiff stain; x 190.

Fig. 7—This chinchilla lung resembles the sheep lung of figure 6, except that there is marked interstitial infiltration. x 190.

Fig. 8—Lung from a Peruvian sheep, with several adenomatous nodules—the multicentric lesion. x 40.

Legends for Illustrations on Opposite Page

Fig. 1—The alveolar walls of a lung of a chinchilla are slightly thickened by macrophagic infiltration. Arrows point to cuboidal epithelium which is beginning to line the alveoli. x 190.

Fig. 2—In this field, there is marked infiltration by lymphocytes which has displaced normal architecture of the lung. x 190.

Fig. 3—An area of interstitial thickening lies to the left, which the adenomatous process approaches from the right. x 190.

Fig. 4—The papillary projection is covered by epithelium taking a deeper stain than that paving the alveolus. Periodic acid Schiff stain; x 380.

Fig. 5—In this small alveolus, the cuboidal-to-columnar proliferative epithelium is seen. x 909.

TABLE 2—Histological Observations in Chinchillas with Pulmonary Adenomatosis

Chinchilla (No.)	Lungs				Other tissues				
	Epithelial proliferation	Alveolar wall thickening	Lymphatic infiltration	Exudative pneumonia	Brain	Digestive tract	Kidney	Liver	Spleen
1	+++	....	+	....	....	....	Tubular casts	FM	Hemosiderosis
2	++	++	+++	+	....	....	....	FM	....
3	+	....	++	....	....	....	....	FM	....
4	++	++	++	+	....	....	....	FM	....
5	+++	++	++	....	....	....	....	FM	....
6	+++	++	++	....	....	....	....	FM	Lymphocytic atrophy
7	++	....	....	....	NPM	....	IN	FM	Lymphocytic atrophy
8	+	++++	+	....	....	....	....	FM	....
9	+++	+++	++	....	....	....	....	FM	....
10	++++	++++	++	....	....	....	....	FM	....
11	+++	++	+	....	....	....	....	FM	....
12	+	....	++	....	....	....	....	FM	....
13	+	++	+	....	....	....	....	FM	....
14	+++	++	+	....	....	....	....	FM	....
15	++++	++++	+	....	....	....	....	FM	....
16	+	....	....	....	....	....	....	FM	....
17	+++	....	....	+	....	....	....	FM	....
18	++++	+	...	++	....	....	Tubular casts	FM	....
19	++	+	+	....	....	....	....	....	....

FM = fatty metamorphosis; IN = interstitial nephritis; NPM = nonpurulent meningitis; +'s indicate the presence and degree of lesions.

while the nephrotic lesions were pale, eosinophilic precipitates in Bowman's spaces and the convoluted tubuli. Parenchymatous degeneration was difficult to ascertain, as this alteration was obvious in all kidneys from chinchillas which were dead on arrival.

DISCUSSION

The criteria of PA, as stated by Liebow,[17] are:

> Under bronchiolar carcinomas shall be included diffuse or focally distributed acinar tumors supported mainly upon the walls of the alveoli, usually bilateral, but slow to metastasize beyond the lung. The peripheral position, probable multicentric origin, relatively benign histological appearance of many of these lesions, and the frequent failure to metastasize, despite massive involvement of both lungs, set them apart from the bronchogenic carcinomas.

Pulmonary adenomatosis of the chinchilla fulfills all these requirements except the "peripheral position," as it did not appear to be confined to any such area. In addition, De Kock [8] and Swan [26] considered jagziekte (PA in sheep) and and PA of man, respectively, true neoplastic diseases. Pulmonary adenomatosis of the chinchilla resembles the disease in other species. For example, histological sections of sheep lungs from Peru revealed that this form was a true oncosis, *i.e.*, little inflammatory reaction accompanied the neoplastic lesions (fig. 6, 8). Examination of material from sheep with chronic progressive pneumonia (CPP)* revealed a pneumonic process; Cowdry and Marshall [5] believed that the adenomatous changes of CPP occurred late in the course of the disease. Pulmonary adenomatosis of the chinchilla more closely resembled the Peruvian form of the disease (fig. 7) and jagziekte than CPP. It likewise resembled PA in man, which bears a close resemblance [1, 2, 12, 16, 21, 22, 25, 30] to jagziekte.

The origin of the neoplastic cell could not be definitely determined. In other species, several possibilities have been suggested: (1) the cells arise from bronchiolar epithelium; [10, 14, 15, 17] (2) the cells arise from alveolar epithelium,[25, 27, 30] which is difficult to see in the mature lung, and the existence of which is denied by some;[12] and (3) the cells are metaplastic and arise from mesodermal elements of the alveolar wall.[16]

In the chinchilla material, it was common to see bronchioles surrounded by radiating lumina lined by the neoplastic cell; all cells were continuous with the bronchi-

* Pulmonary adenomatosis is one of many synonyms.

olar epithelium. Isolated groups of affected alveoli with no obvious connection to bronchioles were seen less often, yet it was possible to visualize this situation as the result of sectioning a peripheral portion of a neoplastic nodule. The authors agreed that the lesion was bronchiogenic in origin. Another influencing fact was that the normal chinchilla lung (fig. 9) did not appear to be lined with a cell of epithelial character.

The cause and pathogenesis of PA in the chinchilla are somewhat nebulous, because the disease has not been reproduced. No causative bacteria have been recovered, but the possibility of a virus agent has not been investigated thoroughly. As for the pathogenesis of the disease, it appears that the interstitial changes of pneumonia are prerequisite to the adenomatous development, as Cowdry[3] observed in jagziekte. In other species, pulmonary irritations were con-

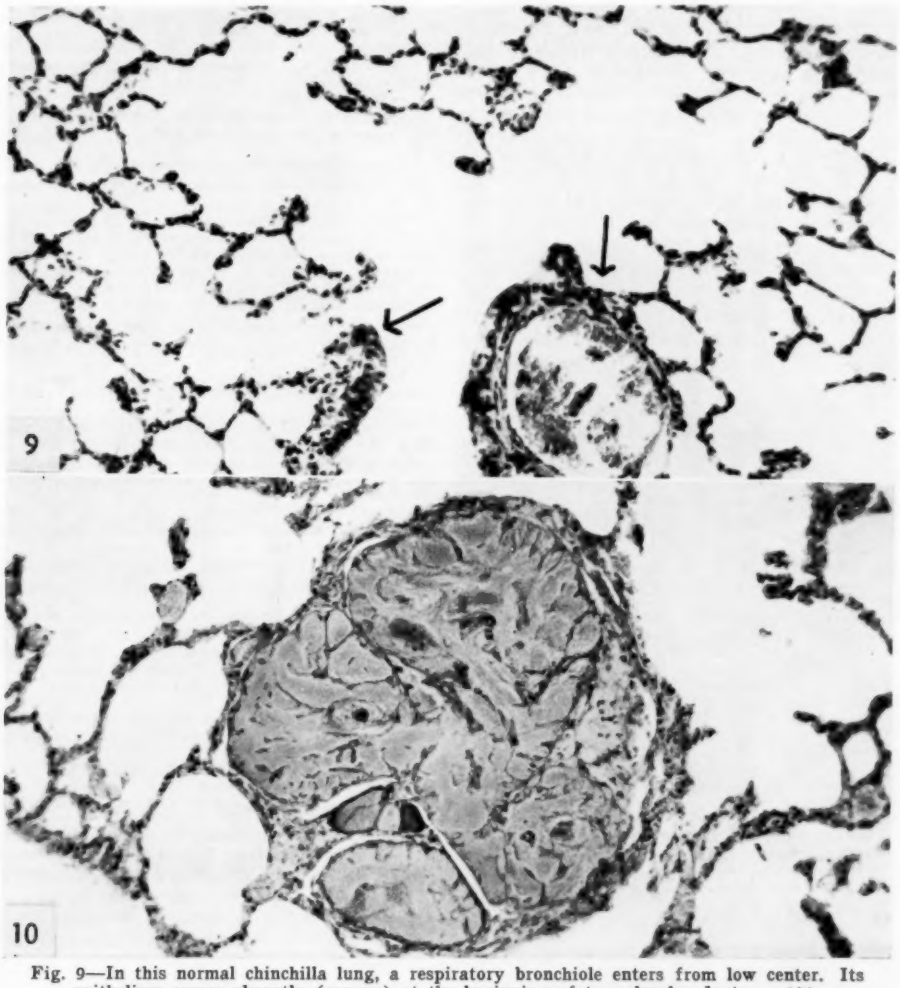


Fig. 9—In this normal chinchilla lung, a respiratory bronchiole enters from low center. Its epithelium ceases abruptly (arrows) at the beginning of two alveolar ducts. x 190.

Fig. 10—Tissue from a female chinchilla which was in late pregnancy when it died. Large trophoblastic cells, weakly positive to periodic acid Schiff stain, fill the picture. One nucleus can be seen in the left portion. x 190.

sidered precursors of PA.[1, 12, 13, 17, 18, 23, 24, 26]

One other pulmonary alteration of the chinchilla also occurs as a bilateral multicentric lesion. This is embolic pulmonary trophoblastosis, which is characterized by the presence of one or more giant cell foci (100 μ or more) scattered about the lung (fig. 10). This appeared only in females with histories of recent pregnancy. Davis reported that these cells arose from the placenta.[7] The same cell was seen in the myometrium, usually at the mucomuscular junction. Wherever seen, these cells failed to provoke any cellular reaction; furthermore, their large size helped differentiate them from PA.

SUMMARY

Pulmonary adenomatosis (PA) was seen in 19 of 206 chinchillas submitted for necropsy. Clinical signs consisted of dyspnea, anorexia, lethargy, and debility; the course of the disease varied from one to six weeks. Gross lesions were not striking. Histological lesions consisted of focal interstitial pneumonia containing alveoli lined by nonciliated cuboidal epithelium. These cells appeared to originate from bronchiolar epithelium.

The histological appearances resembled PA of man and sheep.

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Parakeratosis of the Rumens of Lambs Fattened on Pelleted Feed

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RUMINAL PARAKERATOSIS, a noncontagious disease of sheep, is characterized grossly by hardening and enlargement of papillae and microscopically by the accumulation of excessive layers of keratinized, nucleated, squamous epithelial cells on the papillae.

Casual observation of the rumens of lambs indicated an unusually high incidence of parakeratosis in animals fattened on pelleted feed, a new ration in Colorado, as compared with animals fattened on coarse, nonpelleted feed.

The objectives of this investigation were to determine: (1) the ability of pelleted ration to cause parakeratosis of the rumens of feedlot lambs, and (2) the effect of parakeratosis on rate of gain in body weight.

REVIEW OF LITERATURE

According to Lassiter *et al.*,[7] machines for pelleting feed for livestock were introduced into the United States in 1929. Karstens[5] reviewed the subject of pelleting of feeds and estimated that, in 1955, one third of all manufactured feeds were pelleted, and that 65 per cent of all pelleted feeds were fed to poultry.

Kick *et al.*[6] observed that grinding of feeds to fine consistency decreased the time given to mastication and rumination by steers. Heller *et al.*[3] determined that sheep fattened on finely ground ration consumed less feed, manifested periods of anorexia, and gained less body weight than sheep which were fattened on coarse ration.

TABLE 1—Pelleted Rations for Lot 6 (Experiment 3)

Ingredients	Ration A	Ration B
Alfalfa	40%	60%
Corn	50%	30%
Molasses	10%	10%

Esplin[1] studied the effect of pelleted ration on fattening lambs, and concluded that lambs fattened on pellets made faster and more efficient gains, with a higher dressing percentage, than lambs fattened on nonpelleted feed. Story[9] concurred with Esplin as to the advantages of pelleted rations for fattening lambs. Esplin[2] fattened two lots of lambs on a ration consisting of 47.5 per cent corn, 47.5 per cent alfalfa, and 5.0 per cent molasses. For one lot, the feed was pelleted, while for the other lot it was not. The pelleted feed was more palatable, was consumed in larger amounts, and consequently resulted in more rapid rates of gain in body weight. Carcass grades for lambs fat-

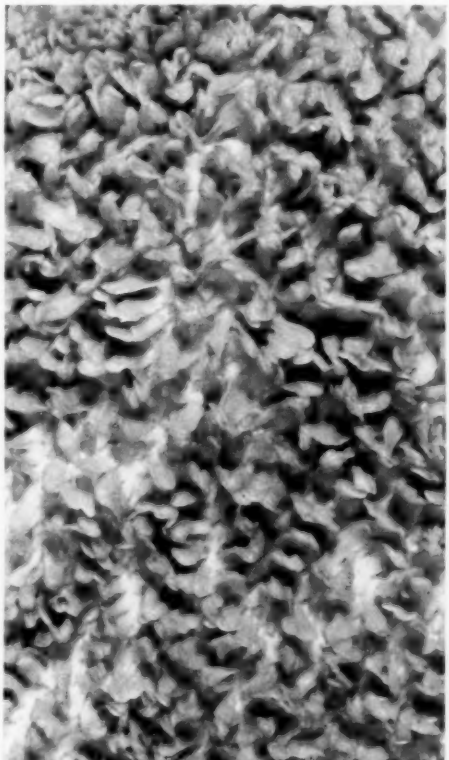

Fig. 1—Normal papillae of the rumen of a feedlot lamb. x 1.5.

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TABLE 2—Effect of Pelleted Feed on the Incidence of Ruminal Parakeratosis in Lambs (Experiment 1)

	Lot 1		Lot 2	
Ration	Alfalfa, ground	40.0%	Alfalfa, chopped	40.0%
	Milo, ground	5.0%	Milo, crackel	5.0%
	Barley, ground	22.5%	Barley, rolled	22.5%
	Corn, ground	22.5%	Corn, cracked	22.5%
	Molasses	10.0%	Molasses	10.0%
Condition of ration	Pelleted		Not pelleted	
Days on feed	65		65	
No. of lambs	50		50	
No. of lambs with parakeratosis of rumen	6		0	

tened on pelleted feed were higher than those for lambs fattened on nonpelleted feed.

Jensen *et al.*[4] observed parakeratosis in the rumens of fattened cattle, to the extent of 8.8 per cent of a random sample of 1,535 cattle. The parakeratosis was thought to be associated with rumenitis. Seghetti and Jensen[6] (in 1956) observed a high incidence of severe, generalized ruminal parakeratosis in slaughtered lambs which had been fattened on a pelleted ration.

MATERIALS AND METHODS

Three experiments were conducted.

Experiment 1.—Crossbreed lambs (100), obtained from a single source, were categorized by sex and weight at the beginning of the experiment. They were then distributed at random to form two equal lots. Fattening began on June 27, 1956, and continued for 65 days. The lambs were again weighed when the experiment terminated.

The ration formula (table 2) was the same for both lots. The ration for lot 1 was pelleted, except for 0.33 lb. of chopped alfalfa per lamb per day, while the ration for lot 2 was not pelleted. For the first few days of fattening, additional alfalfa was given. New feed was provided once daily, and both lots were limited to the same daily intake of feed.

Experiment 2.—Another 100 crossbreed wether lambs, obtained from a single source, were categorized by weight at the beginning of the experiment. They were then distributed at random to form two equal lots (lots 3, 4). The lambs were also weighed at the termination of the experiment.

Ration formulas were identical for lots 3 and 4 (table 3). The ratio of formula concentration to roughage was 1:1 for the first 36 days and 3:2 for the balance of the fattening period. The entire ration for lot 3 was finely ground and pelleted, while the ration for lot 4 was coarse and not pelleted. Feeding began on Nov. 23, 1956, and continued for 58 days. The lambs in both lots were limited to the same daily feed intake.

Following slaughter, lambs of lot 3 (table 4) were put into two categories—lambs with parakeratosis of the rumens, and lambs with normal rumens.

Experiment 3.—This experiment was conducted at a commercial lamb-feeding establishment, using lambs which were owned by the feeder.

Crossbreed lambs (1,569) were arranged, not necessarily at random, to form two lots (lots 5, 6). There were 569 lambs in lot 5, and 1,000 lambs in lot 6.

Lambs in lot 5 were fattened by grazing in fields during the late fall and early winter of 1956 and 1957. They were slaughtered on Jan. 8, 1957. The lambs of lot 6 were fattened on a pelleted ration consisting of corn, alfalfa, and molasses. Ration A (table 1), with 40 per cent rough-

TABLE 3—The Effect of Pelleted Feed on the Rumens of Lambs (Experiment 2)

	Lot 3		Lot 4	
Ration, 1st to 36th days	Alfalfa, ground	50.0%	Alfalfa, chopped	50.0%
	Corn, ground	13.9%	Corn, cracked	13.9%
	Barley, ground	13.9%	Barley, rolled	13.9%
	Millet, ground	13.9%	Millet, rolled	13.9%
	Molasses	8.3%	Molasses	8.3%
Ration, 37th to 58th days	Alfalfa, ground	40.1%	Alfalfa, chopped	40.1%
	Corn, ground	17.2%	Corn, cracked	17.2%
	Barley, ground	17.2%	Barley, rolled	17.2%
	Millet, ground	17.2%	Millet, rolled	17.2%
	Molasses	8.3%	Molasses	8.3%
Condition of feed	Pelleted		Not pelleted	
No. of lambs	50		50	
No. of lambs with parakeratosis of rumen	27		0	
Days on feed	58		58	
Av. gain/lamb/day (in lb.)	0.351		0.389	

age, was used for the first 45 days, while ration B, with 60 per cent roughage, was used for the last 14 days of the fattening period.

At the time of slaughter, the rumen of each lamb was identified. After washing, each rumen was carefully examined for parakeratosis and other abnormalities.

Data on parakeratosis for the lots were analyzed by the *chi*-square method while data on rates of gain in body weight were studied by analysis of variance.

RESULTS

In each of the three experiments, parakeratosis predominated in the rumens of lambs which were fattened on pelleted feed.

Experiment 1.—At the time of slaughter, 6 lambs, all fattened on pellets, showed parakeratosis (table 2). The *chi*-square test of data showed a significant difference ($P<0.05$) for the number of animals which were affected in the two lots. Rates of gain in body weight did not differ significantly.

TABLE 4—Rates of Gain for the Affected and Normal Lambs of Lot 3

	Parakeratosis of rumen	Normal rumen
No. of lambs	27	23
Av. gain/lamb/day (in lb.)	0.3213	0.3889

Experiment 2.—Of the 50 lambs which were fattened on pelleted feed, 27 had ruminal parakeratosis at the time of slaughter. Although the degree of parakeratosis varied, it was generalized and severe in many animals. The *chi*-square analysis showed a highly significant difference ($P<0.01$) between lots for the number of

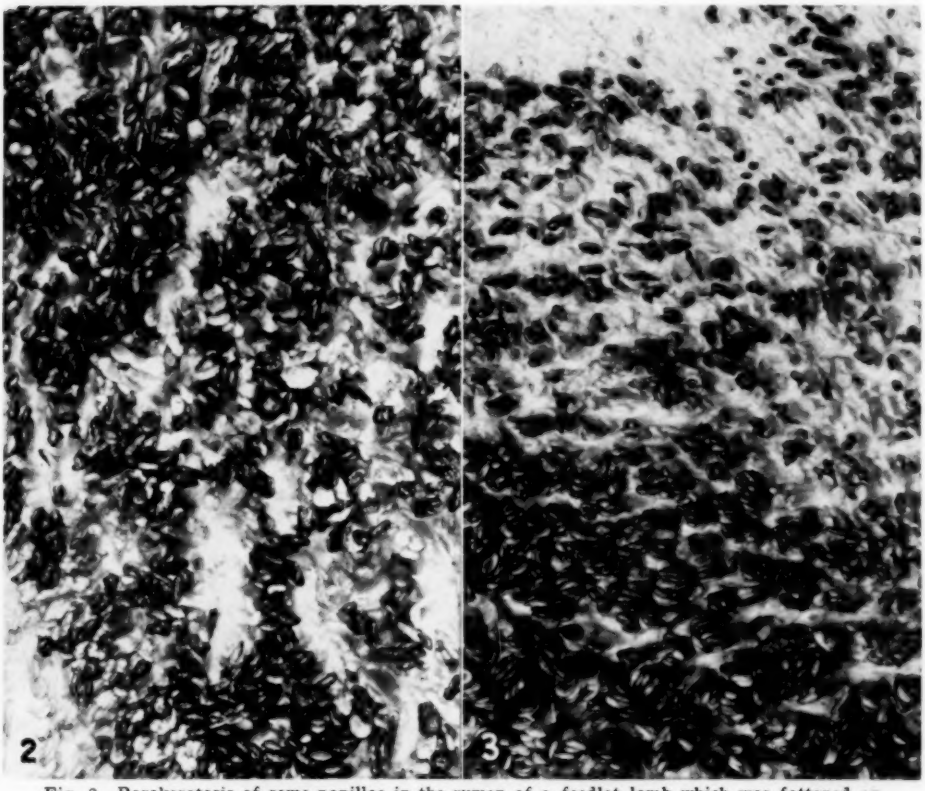

Fig. 2—Parakeratosis of some papillae in the rumen of a feedlot lamb which was fattened on pelleted feed. Affected papillae are darkened and thickened. x 1.5.

Fig. 3—Parakeratosis of some papillae in the rumen of a feedlot lamb which was fattened on pelleted feed. Some papillae are affected throughout most of the surface, while others are affected only at distal ends. x 1.5.

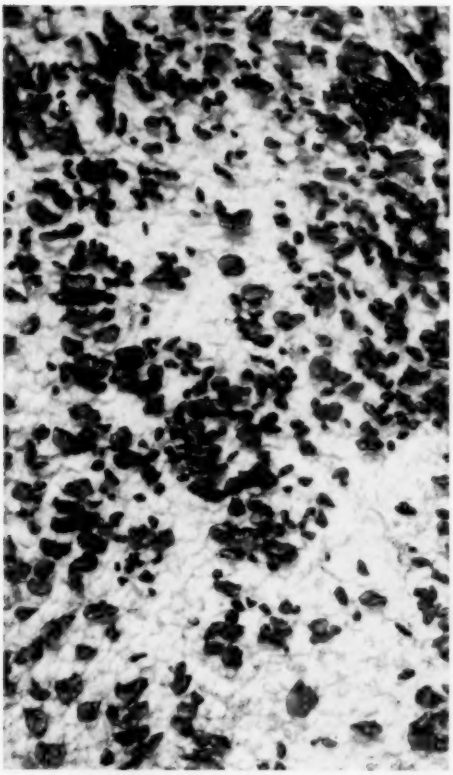

Fig. 4—Parakeratosis of some papillae in the rumen of a feedlot lamb which was fattened on pelleted feed. Affected papillae are dark and firm. x 1.5.

Fig. 5—Parakeratosis of papillae in the rumen of a feedlot lamb which was fattened on pelleted feed. Affected papillae are dark, thickened, and adherent. x 1.5.

animals which were affected with parakeratosis. The rates of gain in body weight for the two lots did not differ significantly ($P>0.05$).

Within lot 3, the lambs which had normal rumens made significantly higher ($P<0.01$) rates of gain than the lambs which had parakeratosis of the rumen (table 4). The initial body weights of the lambs which developed parakeratosis of the rumen did not differ significantly ($P>0.05$) from initial body weights of the lambs which had normal rumens at the time of slaughter.

Experiment 3.—Lambs which were fattened on pellets at a commercial feedlot had significantly ($P<0.01$) more ruminal parakeratosis than animals which were fattened at field pasture (table 5).

Gross- and Histopathology of Ruminal Parakeratosis.—Only the papillae were affected. The number and anatomical distribution varied from a few papillae in the anteroventral sac to most of the papillae on the entire ruminal surface. The highest incidence was in the anteroventral sac. Individual papillae showed parakeratosis over most of the papillary surface or over the surface of the distal part of the papilla only. Affected papillae were firm, leathery, enlarged, and dark in color. In some rumens, affected papillae were adherent, forming clumps. In severe cases, clumps of affected papillae were visible and palpable

TABLE 5—Comparative Effects of Pasture and Pellet Feeding (Experiment 3)

	Lot 5	Lot 6
Ration	Field pasture	A (1 to 45 days) B (46 to 59 days)
Condition of feed	Coarse	Pelleted
No. of lambs	569	1,000
No. of lambs with parakeratosis of rumen	44 (7.7%)	392 (39.2%)

from the serosal surface through the intact ruminal wall. The gross pathological changes are shown (fig. 1–6).

Microscopically (fig. 7, 8), the epithelium was thickened, the malpighian layer was hyperplastic, and excessive layers of keratinized, nucleated cells had accumulated on the surface. Colonies of bacteria and ingesta adhered to the surface.

DISCUSSION

Although the specific cause of ruminal parakeratosis is not known, the causative factors were contained in the pellets. Three possible etiological factors were: (1) finely ground feed which may have predisposed by creating a medium for bacteria to produce causative agents; (2) contaminants from machinery which may have entered the feed at the time of pelleting; and (3) deficiencies which may have resulted from alteration of ingredients by processing.

The initial changes, which terminated in the manifestation of parakeratosis, may

Fig. 6—Parakeratosis of a single papilla from the rumen of a feedlot lamb which was fattened on pelleted feed. Only the distal half of the papilla is affected. x 6.

Fig. 7—Parakeratosis of papilla from the rumen of a feedlot lamb which was fattened on pelleted feed. Epithelium is thickened, especially in stratum germinativum and stratum corneum. H & E; x 86.

Fig. 8—Parakeratosis of ruminal papilla from a lamb which was fattened on pelleted ration. Excessive layers of cornified epithelium have accumulated on the surface. H & E; x 86.

have been inflammatory and caused by irritants from fermentation during periods of adaptation to a fattening ration. The adverse effect on rates of gain in body weight was demonstrated by lambs of lot 3, and may have resulted from interference to absorption through the ruminal mucosa.

Ruminal parakeratosis also occurs in fattened cattle and prevents efficient cleaning and bleaching of the rumen in the preparation of tripe. In cattle, the effect on rates of gain in body weight has not been demonstrated.

SUMMARY

Ruminal parakeratosis is a noncontagious disease characterized, grossly, by hardening, enlargement, and clumping of mucosal papillae and, microscopically, by accumulation of excessive layers of keratinized, nucleated, squamous epithelial cells on the papillae. In each of three experiments, lambs fattened on pelleted feed showed significantly more ruminal parakeratosis at the time of slaughter than lambs fattened on the same feed ingredients in the coarse state.

Lambs which had normal rumens showed a significantly higher rate of gain in body weight than lambs which had ruminal parakeratosis.

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The Vaginal Cytology of the Ewe During the Estrous Cycle

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ESTROGENS, natural or synthetic, have a direct effect on the vaginal epithelium of most female mammals. Changes occur in an orderly sequence and can be followed by studying vaginal smears taken at regular intervals.

An increase in estrogens in the body stimulates an increase in the number of cell layers lining the vagina, mobilizes glycogen in the cells, and causes cornification of the superficial layers. As the estrogen level diminishes, extensive desquamation of the superficial layers follows and, as the estrogen increases again, the cycle is repeated.

These phenomena were observed in a study of vaginal smears taken during normal estrous cycles from 2 ewes and are reported in this paper. Additional ewes were used in another study.[7]

REVIEW OF LITERATURE

Grant,[3] Dukes,[2] Radford and Watson,[5] and Cole and Miller[1] studied vaginal smears in the ewe. In general, during proestrus, they found thick mucus, leukocytes, and a few epithelial cells; during estrus, mucus was thin and clear, and a few cornified, squamous cells and leukocytes were seen. In both metestrus and diestrus, mucus was thick and cheesy, masses of cornified squamous cells were found, and some leukocytes were seen. The mouse, rat, guinea pig, cow, sow, baboon, monkey, and opposum have also been studied.[7]

Papanicolaou[6] described five cell layers in the vaginal wall of the human female, and the cells found in vaginal smears are derived either from these layers or from the ectocervix. The deepest, or basal zone, is a single layer of cuboidal or columnar cells with a relatively high nuclear-cytoplasmic ratio. Cells of this layer are not shed normally and usually are not found in a smear.

The next layer, the parabasal zone, is made up of several layers of round, oval, or polyhedral cells with fairly large nuclei and intercellular bridges. The outer cells of this layer are frequently found in smears.

The third layer, the intermediate zone, consists of cells with a navicular form. These are smaller and have larger nuclei than superficial squamous cells, from which they can be differentiated by their form.

Cells of the fourth layer can not be distinguished from the superficial squamous cells in smears.

The fifth layer, the superficial zone, consists of large, flat, sometimes folded cells, with an irregular or polygonal form and small pyknotic nuclei. Some of the cells are basophilic (precornified), while those undergoing cornification are acidophilic. The ratio of basophilic to acidophilic cells depends chiefly upon the estrogenic hormonal level.

MATERIALS AND METHODS

Two healthy, yearling, virgin Columbia ewes, on bluegrass pasture, were used in this study. The wool around the external genitalia and tail was closely clipped. For collecting the smears, the ewes were restrained on the left side, the external genitalia were thoroughly cleaned with detergent and warm water, rinsed, and dried. A sterile glass tube (⅝ by 6 in.) was inserted full-length into the vagina, through which an 8-inch glass rod was passed. The rod was manipulated so as to collect a maximum amount of cells, detritus, and mucus from the anterior fornix. A thin smear was quickly made on a clean glass slide and dropped into equal parts of 95 per cent alcohol and ether for immediate fixation and to prevent drying. Smears were collected daily through one complete cycle for each ewe during September and October. The exact time of estrus was established by the use of aproned rams.

All slides were stained by the Papanicolaou[6] method. In this stain, the basophilic cells were stained shades of green varying from light to dark, depending upon the age of the cell and the origin. Cervical epithelium and the deeper layers (less mature cells) of the vagina stained a deep shade of green, while the more mature, superficial squamous cells of the vagina stained only light green. Early cornification of cells was indicated by the presence of small areas of magenta-colored cytoplasm around the nucleus, while the periphery remained green. Complete cornification had occurred when the entire cytoplasm was stained pink to red (eosinophilic). Bacteria were stained green; mucus, pale blue; and leukocyte nuclei, blue-black. Dense cornified cells and hyalinized cells were bright orange, while fully keratinized, non-nucleated cells were bright, transparent yellow. The description of the cell layers from which cells came, as well as the cellular morphology, given by Papanicolaou[6] was followed in this work.

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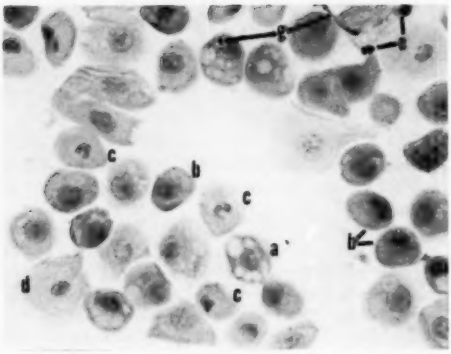

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Fig. 1—Vaginal smear of ewe during proestrous phase. Only small numbers of cells were present, and these were basophilic-staining: (a) some cells had vacuoles in the cytoplasm; (b) cell with magenta-colored cytoplasm around nucleus, with basophilic cytoplasm forming the border of the cell (early cornification); (c) karyorrhexis and pyknosis of nuclei; (d) pale pink, cornified cell; (e) neutrophil and monocyte; (g) artefacts. Papanicolaou's stain; x 300.

RESULTS AND DISCUSSION

Proestrus.—Little material was visible on the glass rod until 24 hours before estrus, at which time there was an increase in clear, thin mucus. On this stained smear, no cells were found. On the smear taken 48 hours before estrus, a few cells were present (fig. 1). All cells were the large, flat, squamous type from the superficial layers and were basophilic-staining, although some had pale, faded green cytoplasm encircling the nucleus, leaving a dark green border at the edge of the cell. Other cells had a magenta-colored cytoplasm surrounding the nucleus, with a dark green fringe at the cell border. Both of these conditions were interpreted as stages of early cornification. The cells were distinct, well separated, and had sharp borders; most had large, well-stained nuclei. However, a few nuclei were undergoing karyorrhexis or pyknosis. A few cells contained small, clear vacuoles in the cytoplasm. An occasional neutrophil or lymphocyte was seen among the cells, and small strands of pale blue-stained mucus and bacteria were present.

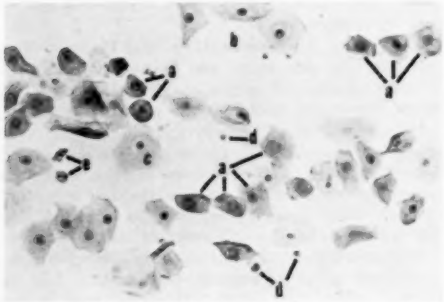

Fig. 2—Vaginal smear of ewe during estrous phase. Only a few scattered cells were present and most of these were (a) basophilic squamous cells; (b) three cornified squamous cells, with perinuclear vacuole in one; (c) large, pale green (basophilic) cell with small ring of eosinophilic cytoplasm around the nucleus (early cornification); (d) neutrophils; (e) artefacts. Some of the cornified cells had curled edges and slightly distorted outlines. Papanicolaou's stain; x 172.

Estrus.—During estrus, mucus was thin, clear, and copious. It clung to the rod, had a tendency to pull on the slide, formed long threads when the rod was lifted from the slide, and contained small air bubbles.

All of the cells were of the superficial squamous type (fig. 2), about two thirds of which were basophilic; the remainder were eosinophilic, indicating cornification. Cells with the magenta-colored cytoplasm around the nucleus, as described for the proestrous smear, were not present in the estrous smear; however, nearly all cells had rolled edges or irregular shapes. Nuclei remained distinct, centrally located, and well-stained. A few cells had undergone complete keratinization and were bright yellowish orange, had pyknotic nuclei and irregular shapes. According to Papanicolaou, a few cornified cells can be found in all phases of the estrous cycle; therefore, the presence of a few of these cells can be expected. Slight mucus, a few bacteria, and a few neutrophils were present.

Metestrus.—Twenty-four hours following estrus, the smear consisted of clear mucus, although it was much thicker than during estrus. After 48 hours, the material collected on the rod was thick, white, cheesy, and sticky. Because of these characteristics, smears were dense and cell masses were several cells thick. During metestrus and diestrus, the vagina must contain large amounts of this material, since it was present throughout these two phases in large quantities. The glass tube was also partially filled with it each time it was inserted.

In reviewing the literature, it was found that many authors referred to this material

as white, cheesy mucus. However, this appears to be inaccurate, because mucus is not of this nature and, on microscopic examination of the stained smears, very few mucous strands were present. Instead, this material was composed of great numbers of bacteria, masses of desquamated, cornified epithelial cells, leukocytes, and detritus.

Cells formed clumps and tended to cling to each other (fig. 3), rather than spreading out individually, as in the proestrous and estrous smears. An occasional large, flat, basophilic, squamous epithelial cell from the vagina, and a few small, round, dense, basophilic cells from the cervix, were scattered among the cornified cells. Some of these small cells showed early cornification of the cytoplasm around the nucleus, and a few had pyknotic nuclei.

The cornified cells were of two types. One was a small, round, dense, and deeply orangeophilic cell, with a centrally located pyknotic nucleus. These cells were scattered throughout the slide and intermingled with the other cells. They had a sharp border and a bright, hyalinized appearance. These cells were from the deeper layers of the vaginal wall and corresponded to Papanicolaou's parabasal cells. According to Papanicolaou,[4] these cells are characteristic of high estrogenic activity or pregnancy. In this study, pregnancy was not a factor.

The other type of cornified cell was the large, flat, pale, squamous type from the superficial layers of the vagina. These cells had a tendency to form clumps and they stained a bright eosinophilic color. A few showed faint traces of green in the cytoplasm. These cells had curled and folded edges, were wrinkled, and a few were irregular in shape. Most cells contained nuclei which were centrally located, pale, round to oval, and many were pyknotic, but a few showed karyorrhexis. An occasional hyalinized epithelial pearl was found.

Bacteria were numerous; neutrophils were present, both singly and in groups; and mucus was scant.

Diestrus.—During this phase, the vagina was dry and resisted passage of the glass tube. Only thick, white, cheesy material was collected on the rod and this material partially filled the tube as it was inserted.

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Fig. 3—Vaginal smear of ewe during metestrous phase. Notice tendency for cells to clump and cling together. The smear was heavy and almost all cells were cornified. Basophilic-staining cells from the vagina (a) and from the ectocervix (b) were infrequent; (c) small, round, dense, cornified cells from the parabasal zone, characteristic of high estrogenic activity; (d) groups of large, flat, completely cornified cells, many with curled edges, folded and irregular shapes. Papanicolaou's stain; x 247.

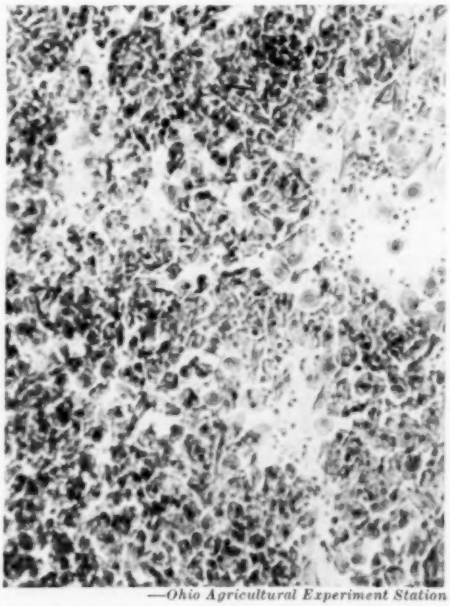

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Fig. 4—Vaginal smear of ewe during diestrous phase. Notice extremely thick smear. Almost all cells were cornified; many had curled edges, irregular shapes, and some showed loss of nuclei, as well as other degenerative changes. Neutrophils were numerous and appeared as small black bodies. Bacteria were present in large numbers. Papanicolaou's stain; x 150.

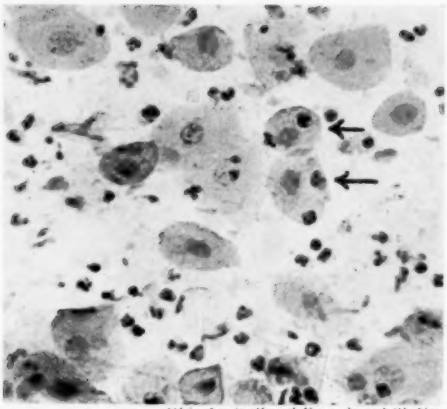

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Fig. 5—Vaginal smear of ewe during diestrous phase. Notice neutrophils which appeared to be inside the epithelial cells (arrows). This was considered accidental and not evidence of phagocytosis. Papanicolaou's stain; x 344.

The most striking features of the smears from this phase were the extremely thick masses of epithelial cells and the heavy infiltration of neutrophils (fig. 4). Most of the cells were cornified and were of the two general types described for the metestrous smear. The small, round, bright, orangeophilic cells were numerous and were found throughout the slide. The large, flat, cornified squamous cells were present in thick masses, large clumps, and in piles many cells deep. Most of the cells were well preserved and evenly stained, with centrally located nuclei, although folded edges and wrinkled cells were prominent and many cells showed evidence of degeneration. This was characterized by ragged edges, partial loss of cytoplasm, and faint nuclei. Occasional cells had lost the nuclei. A few bright yellow, non-nucleated, fully keratinized cells were present.

Some cells appeared to have been invaded by neutrophils (fig. 5). This was considered by Papanicolaou to be accidental and not evidence of phagocytosis. Bacteria were present in large numbers throughout the slide and occasional mucous strands could be seen.

As the diestrous phase progressed toward proestrus, there was a gradual change in the character of the smear. The white, cheesy material gradually decreased in amount, desquamated cornified cells were less numerous, and the basophilic-type cells gradually increased in number and mucous strands were more abundant. Total cell numbers decreased and the smear became thinner until, at the end of the diestrous phase, little material adhered to the glass rod and slides appeared to have only faint streaks of material deposited on the surface.

There seemed to be a gradual transition from diestrus to proestrus and from proestrus to estrus which could be followed on both the gross and microscopic smears. However, the transition from estrus to metestrus was abrupt and the change in the character of the smear was marked. This was evident both grossly and microscopically. The transition from metestrus to diestrus was not as well defined. Grossly, there was little to indicate the transition, other than counting calendar days. Microscopically, the primary difference between the two phases were characterized by thicker masses of cells, more evidence of cellular degeneration, and greater numbers of bacteria in the diestrous phase.

The extensive cornification and desquamation of epithelium which occurred in metestrus and diestrus followed a rise and fall of the level of estrogen in the body. Radford [6] ovariectomized ewes and studied vaginal smears from these ewes. He found that these phenomena did not occur in the absence of estrogen but, immediately following the administration of estrogen, cornification and desquamation appeared. Sanger *et al.*[7] found a similar occurrence in anestrous (nonovariectomized) ewes which were studied on another project. Grant [3] studied anestrous smears of normal ewes and found the smears to be monotonously similar from day to day, with small quantities of mucus; a few healthy epithelium cells were usually present. Keratinized cells were rarely found and leukocytes were found less frequently than during the breeding season.

The vaginal cytology of ewes during a normal estrous cycle follows a regular sequence which is similar to that for many other animals which have been studied.

SUMMARY

The vaginal cytology of 2 ewes during normal estrous cycles was studied, using the Papanicolaou staining technique. An aproned ram was used to establish the presence of estrus.

In proestrus, only small groups of large, flat, squamous basophilic cells were present. Some showed early cornification. Cells were distinct and well separated.

In estrus, only a few cells were found on the slide. These were squamous in type, and about two thirds were basophilic and one third acidophilic (cornified). A few showed folds, wrinkles, and curled edges. All cells were separate and distinct.

In metestrus and diestrus, smears were thick and sticky; cells were clumped or piled up, and nearly all cells were cornified. Neutrophils and bacteria were abundant.

A marked difference was present between the cytology of the diestrous and proestrous phases, between the proestrous and estrous phases, and between the estrous and metestrous phases of the estrous cycle in the ewe. Much less difference was apparent in the cytology between the metestrous and diestrous phases.

The phases of the estrous cycle in ewes can be followed by studying the character and cytology of the vaginal smears.

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Evidence of Estrogenic Stimulation in Anestrous Ewes Pastured on Ladino Clover and Birdsfoot Trefoil, as Revealed by Vaginal Smears

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THE PRESENCE of estrogens in the system of a normal, mature female animal is accompanied by characteristic changes in the reproductive tract. The vaginal epithelium is markedly responsive to estrogens, and the changes which occur in these epithelial cells can be followed by studying vaginal smears. Both natural and synthetic estrogens cause these changes and, apparently, the feeding of plants which contain estrogen-like substances will also produce these changes.

For several years, extended lambing seasons have been observed in the sheep-breeding flocks of the Ohio Agricultural Experiment Station when they were pastured on lush Ladino clover and birdsfoot trefoil plots.

It was suspected that some substance, possibly estrogenic in nature, was present in these plants, which was interfering with conception.[8, 9]

Qualitative evidence of the presence of estrogen-like activity in Ladino clover and birdsfoot trefoil is presented in this paper.

REVIEW OF LITERATURE

Bennetts *et al.*[3] related female sterility, dystocia, and uterine prolapse in ewes to grazing on subterranean clover in Australia, and Curnow *et al.*[6] extracted a substance which produced uterine and vaginal changes qualitatively similar to those produced by the injection of estradiol, a known estrogen.

Engle *et al.*[9] reported irregular and prolonged lambing periods in flocks of ewes grazed on Ladino clover and birdsfoot trefoil pastures during three breeding seasons; however, sterility, dystocia, and uterine prolapse were not a problem. Engle[9] succeeded in extracting an estrogen-like substance from Ladino clover and birdsfoot trefoil, but could not recover it from bluegrass. When this material was injected into immature female mice, a significant increase in uterine weight occurred. In the controls, an increase in uterine weight also followed the injection of estrogens into immature female mice.

Numerous workers have described the cytological changes which occur in the vaginal epithelium of many different animals during the successive phases of the normal estrous cycle. In the guinea pig, Stockard and Papanicolaou[28] found normal epithelial cells and many leukocytes in estrus, and large numbers of leukocytes and atypical, desquamated epithelial cells during diestrus. In vaginal smears from the rat, Long and Evans[14] described many small, round, normal cells, thick, cheesy mucus, and leukocytes during metestrus, and only a few epithelial cells and many leukocytes during diestrus.

In mice, Allen[1] and Robson[21] found well-defined, nucleated epithelial cells and thin, clear mucus in proestrus; cornified cells with indistinct nuclei and no mucus during estrus; cornified cells, sticky mucus, leukocytes and detritus in metestrus; and few to many small, epithelial cells, thick mucus, and neutrophils during diestrus. Hartman[13] found, in the opossum, that the smears varied only in the degree of cornification through the estrous cycle, with leukocytes present at metestrus.

In the sow, Wilson[24] did not describe cells of the proestrous phase but, during estrus, degenerating epithelial cells, thin, clear mucus, and leukocytes were found. In metestrus, the number of epithelial cells increased and scattered leukocytes were present. In diestrus, true cornification was not seen, mucus was thick and a few leukocytes were found.

In the cow, Frie and Metzger[10] found masses of detritus, many leukocytes, cornification, and degeneration in proestrus, while during estrus, leukocytes were absent but debris and cornified and degenerating cells were found. Through metestrus and diestrus, masses of desquamated cells undergoing degeneration, and a marked increase in leukocytes were seen. Allen[2] found only erythrocytes and masses of leukocytes during proestrus in the monkey. Through estrus, metestrus, and diestrus, only cornified cells were found.

Cole and Miller,[5] Grant,[12] Dukes,[7] and Radford and Watson[20] examined smears from sheep and found thick mucus, leukocytes, and a few epithelial cells during proestrus. During estrus, mucus was thin and clear, some cornified squamous cells were present, and a few leukocytes could be found. During metestrus and diestrus, mucus was thick and cheesy, masses of cornified, desquamated cells were present, and some leukocytes were seen.

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Sanger *et al.*[22] studied vaginal smears from ewes, taken during a normal estrous cycle. In proestrus, the smear was thin and clear and cells were scarce. All cells were from the superficial layers of the vagina and were basophilic-staining. In estrus, the smear contained more mucus, cells were scarce and of the superficial type, and both basophilic and eosinophilic cells were present. In metestrus and diestrus, the smear was thick, sticky, white, and cheesy; both superficial and parabasal cells were present. Nearly all cells were cornified; many had curled edges or were folded. Some cells showed evidence of degeneration. In proestrus and estrus, an occasional leukocyte was seen, while in metestrus and diestrus, both leukocytes and bacteria were numerous.

Radford[19] ovariectomized ewes, after which he found the vaginal smear to be serous or mucous in nature, but never cheesy. However, within a few days following the administration of estrogen, thick, cheesy material appeared and persisted from one to 12 days. The cytology of these smears was not described, but the gross consistency of the material from ovariectomized ewes which had received estrogen was apparently similar to that for the metestrous and diestrous phases of a normal estrous cycle described by numerous workers.

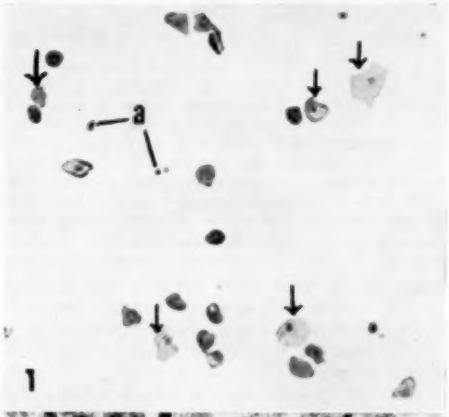
Gillman[11] studied the baboon and found a few small cells, mucus, and leukocytes during proestrus. During estrus, metestrus, and diestrus, smears were heavy and contained cornified, wrinkled, clumped cells, white cheesy mucus, and some leukocytes. In the gorilla, Norback[15] found a few superficial cells and many neutrophils during proestrus. During estrus, there were many superficial basophilic cells and a few leukocytes, while during metestrus and diestrus, a mixture of basophilic and acidophilic cells were found. He also stated that the cyclic changes in the cellular contents of the vaginal smear from the gorilla were similar to those reported for other primates, including man.

Papanicolaou[16,17] and Papanicolaou, Traut, and Marchetti[18] studied vaginal smears from the human female and found, during proestrus (menstrual phase), many erythrocytes, a few endometrial cells, many neutrophils, and both basophilic and acidophilic epithelial cells. During estrus (follicular phase), there was an increase in cornified cells with pyknotic nuclei and high glycogen content; perinuclear vacuoles and leukocytes had invaded desquamated cells but the smear was clear and the staining reaction was good. In metestrus (proliferation phase), both basophilic and acidophilic cells were present, but most cells were cornified with curled edges, irregular outlines and some folding. The smear was dry and leukocytes were present. During diestrus (growth and secretory), navicular-type cells with some folding and a tendency toward smaller grouping were seen; neutrophils were present and basophilic cells were increasing.

MATERIALS AND METHODS

For these studies, four pairs of virgin, yearling Columbia ewes were selected. All animals were closely clipped around the tail and external genitalia. The method of restraint and preparation of the ewes and collection, fixing, and staining of the smears were described in another paper.[20]

Smears were collected daily from all ewes. Two pairs served as controls. One pair was penned in the barn during July, fed dry feed, and smears were collected for seven days, following which two injections of 0.25 mg. of estradiol were given intramuscularly (48 hours apart) and smears were collected for another seven days. Smears from the



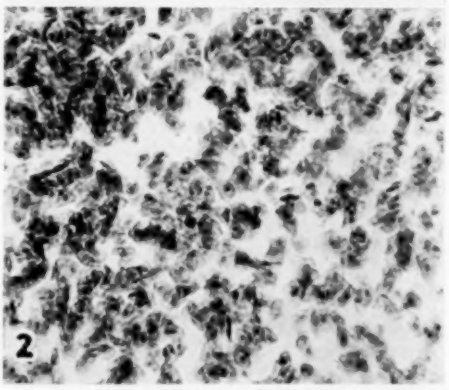


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Fig. 1—Vaginal smear from control ewe, anestrous phase. Notice scarcity of cells. Parabasal and squamous cells were present; all cells were intact, distinct, and basophilic-staining, except a few which were cornified (arrows); (a) neutrophils. Papanicolaou's stain; x 140.

Fig. 2—Vaginal smear from the same control ewe, taken 96 hours after injection of estradiol. The smear was thick, desquamation was heavy, and all cells were cornified. Notice tendency to clump, rolled edges, folded cells, and pyknotic nuclei. Papanicolaou's stain; x 98.

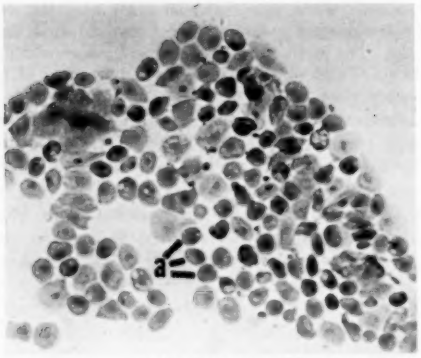

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Fig. 3—Vaginal smear from control ewe, proestrous phase. Only small, scattered groups of cells were present. All cells were basophilic-staining, with a few (a) showing a small ring of magenta-colored cytoplasm around the nucleus, indicating early cornification. A few neutrophils were present; some cells had pyknotic nuclei, and a few had small vacuoles in the cytoplasm. Papanicolaou's stain; x 150.

other control pair were collected for an entire normal estrous cycle in September and October.

The third pair was pastured continuously on Ladino clover and the fourth pair continuously on birdsfoot trefoil. Smears were collected for 11 consecutive days during anestrus in August. The presence or absence of estrus was checked by the use of aproned rams.

RESULTS

Anestrous Control Ewes.—Anestrous smears taken before the injections of estrogens were scanty, with only slight mucus (fig. 1). Cells were not numerous, but both parabasal and squamous cells were present. Nearly all of the cells were basophilic, indicating low estrogenic activity; some had irregular shapes and others had vacuoles in the cytoplasm. An occasional cornified and orangeophilic cell was seen. Scattered neutrophils were present, but bacteria were absent.

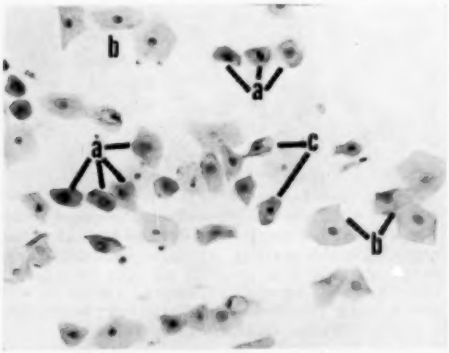


Fig. 4—Vaginal smear from control ewe, estrous phase. The smear was scant and consisted of basophilic (a) and eosinophilic (b) superficial squamous cells. Some of the cornified cells had pyknotic nuclei and irregular shapes (c). A few neutrophils were present. Papanicolaou's stain; x 172.

Following the injection of estrogens, there was swelling of the vulva and copious amounts of mucus appeared, after which, smears became heavy, thick, and cheesy. Nearly all cells were cornified, many having curled edges, irregular shapes; some had navicular forms, pale nuclei, and numerous orangeophilic types were found (fig. 2). Only a few basophilic parabasal cells were present; leukocytes and bacteria were absent. The character of the smears did not change noticeably over the seven days.

Control Ewes, Normal Cycle.—Proestrous smears (fig. 3) revealed scant mucus with basophilic, parabasal, and squamous cells, some of which had vacuoles in the cytoplasm. There were scattered cornified cells, a few neutrophils, but no bacteria.

The estrous smear was scant (fig. 4) and mucus was thin and clear. Basophilic, parabasal, and squamous cells were numerous, but cornified squamous cells were increasing in number when compared with the proestrous smears. An occasional neutrophil was seen, but bacteria were absent.

The metestrous smear was heavier; it was thick, with a few basophilic, parabasal, and squamous cells, but most of the cells were large, discrete, pale, cornified, squamous cells (fig. 5). A few neutrophils and large numbers of bacteria were present.

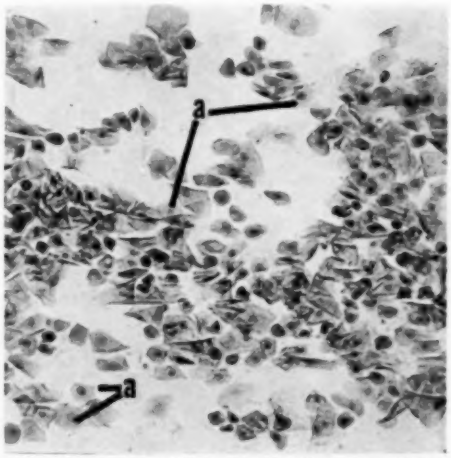
The diestrous smear was heavy (fig. 6); the exudate was dry, cheesy, and white. Early in diestrus, there was desquamation of a few basophilic epithelial cells, with small areas of cornification in the cytoplasm, usually around the nucleus. These were soon lost and the heavy smear consisted almost entirely of cornified squamous epithelium, with cells showing pyknosis, karyorrhexis, rolled edges, irregular shapes, and clumping. Many orangeophilic non-nucleated cells were present. As proestrus approached, more basophilic cells again appeared. Neutrophils, lymphocytes, and bacteria were numerous.

Ewes on Ladino and Birdsfoot Trefoil.—

All smears were taken during anestrus, and the smears from all 4 ewes were remarkably similar, except that the cellular changes of the smears from the ewes on Ladino were slightly more marked. All the slides will be described as a group.

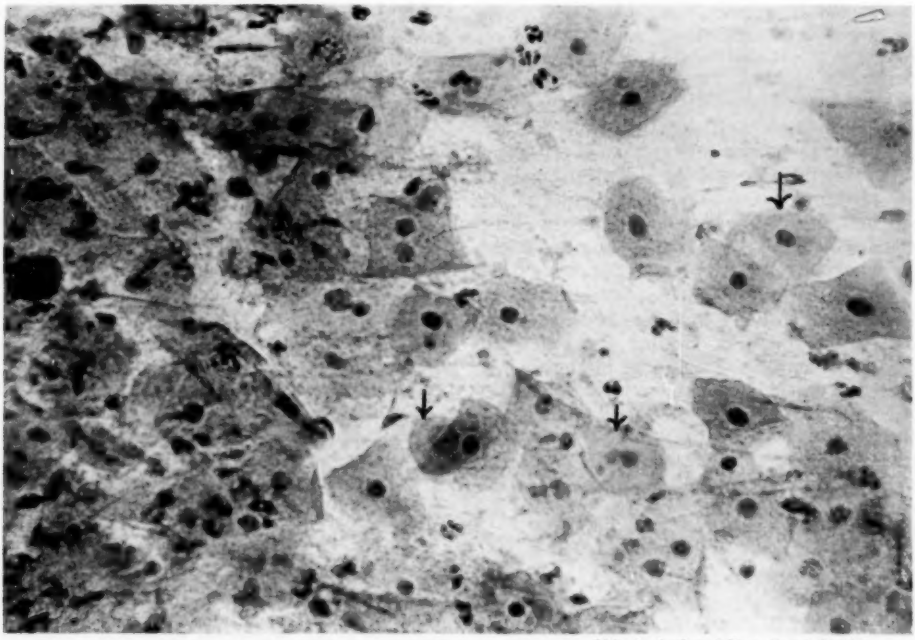
Smears were consistently heavy and thick. The material was cheesy, had a whitish color, and varied from moist to semidry. Frequently, there was dried exudate on the vulva.

The smear consisted almost entirely of masses of large, pale, cornified, squamous cells which were clumped or single, with karyorrhexis or pyknosis of many of the nuclei (fig. 7, birdsfoot trefoil; fig. 8, Ladino clover). Some cells were wrinkled; others had folded edges. Perinuclear vacuoles were present in some cells. There were numerous bright yellowish, non-nucleated cells which had ragged, indistinct edges and irregular staining qualities; nuclei were absent. Neutrophils, lymphocytes, and bacteria were abundant. Only occa-



—Ohio Agricultural Experiment Station

Fig. 5—Vaginal smear from control ewe, metestrous phase. Notice thick smear, clumping of cells, with many showing curled edges and folding. Only scattered basophilic cells (a) were present; all other cells were cornified. Bacteria and neutrophils were present. Papanicolaou's stain; x 150.



—Ohio Agricultural Experiment Station

Fig. 6—Vaginal smear from control ewe, diestrous phase. Notice the heavy smear, clumped cells at the left, curled edges, folded cells, karyorrhexis, and pyknosis of nuclei. Three basophilic cells (arrows) were present; all others were cornified. Leukocytes were numerous and bacteria were so numerous as to cause a pale greenish background. A few strands of mucus can be seen. Papanicolaou's stain; x 437.

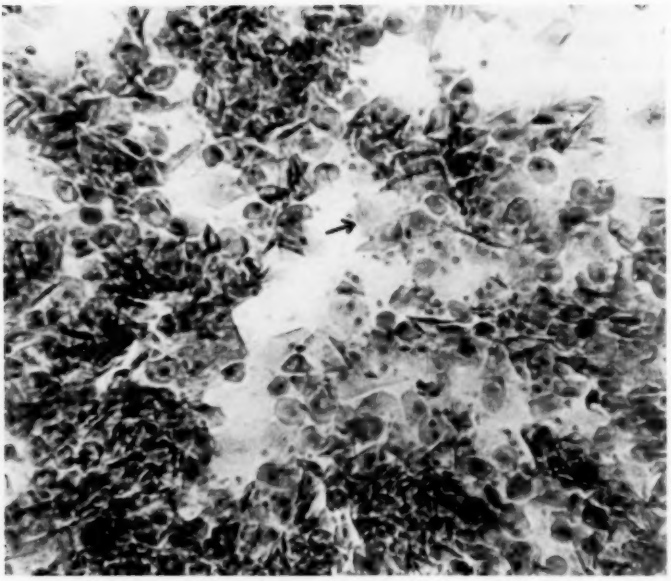


Fig. 7—Vaginal smear from ewe on birdsfoot trefoil, anestrous phase. The smear was thick; cells were clumped and many had curled edges, were folded, and had pyknotic nuclei. All cells were cornified except one (arrow). Neutrophils, lymphocytes, and bacteria were thick. Papanicolaou's stain; x 216.

sional small, round, basophilic, parabasal or large, flat, distinct, squamous cells were found.

DISCUSSION

Cornification and stratification are two essential responses of vaginal epithelium to estrogen.[4] As soon as essential levels of the hormone decrease, desquamation of the epithelium occurs. In the absence of estrogens, as during anestrus, these phenomena do not occur.

In his studies of anestrous smears of normal ewes, Grant [12] found that the smears were monotonously similar from day to day, with small quantities of mucus; a few healthy, epithelial cells were usually present. Keratinized cells were rarely found and leukocytes were found less frequently than during the breeding season. Anestrous smears from the control ewes in these studies were similar in nature to those described by Grant. However, soon after the injection of estrogen, a marked increase in numbers of desquamated cells occurred and smears became extremely thick and cheesy. Almost all cells were now cornified. The gross appearance of these smears resembled those described by Radford.[19]

The same type of thick smear was found during metestrus and diestrus from control ewes during normal estrous cycles. Nearly all cells were cornified and leukocytes were more numerous. Near the end of the diestrous period, as proestrus and estrus approached, the smears changed and became much thinner, to the extent that it sometimes appeared as if no cells were obtained on the smear. Small amounts of

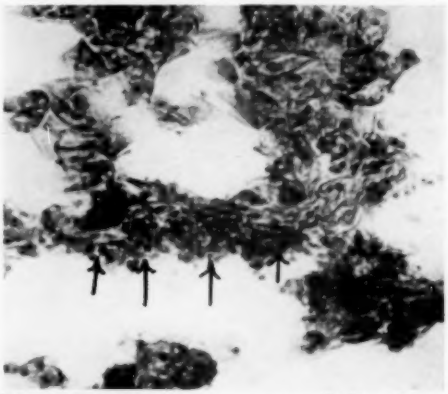


Fig. 8—Vaginal smear from ewe on Ladino clover, anestrous phase. Notice the thick smear, clumped cells, and cells with curled edges, folds, and faint nuclei. All cells were cornified. Masses of neutrophils which appeared as black dots (arrows) and bacteria were intermingled with the cells. Papanicolaou's stain; x 199.

clear mucus were present. Examination of these stained slides revealed smears resembling those seen during anestrus. Scattered, normal basophilic cells and only a few leukocytes were present.

Since Engle[9] was able to extract an estrogen-like substance from Ladino clover and birdsfoot trefoil, it was postulated that vaginal smears from anestrous ewes pastured on these legumes should show effects of estrogen activity, even though the normal body level during anestrus is too low to cause cornification and desquamation.

Both grossly and microscopically, smears from ewes on both types of pasture closely resembled those from the anestrous control ewes which had been given estrogens and the metestrous and diestrous smears of a normal estrous cycle from the control ewes. The smears were thick, sticky, and white, with large amounts of material present. Microscopically, the smears consisted mainly of cornified cells, many leukocytes, bacteria, and cellular debris.

When smears from the ewes on Ladino clover and birdsfoot trefoil were compared, they were similar in all respects, except that cell distortion, degeneration, and clumping were more noticeable from the ewes on Ladino clover. This could be interpreted to mean that Ladino clover contained greater amounts of the estrogen-like substance than birdsfoot trefoil. Also, Engle *et al.*[8] found a slightly greater variation in conception and lambing from ewes on Ladino than from those pastured on birdsfoot trefoil, and larger quantities of the estrogen-like material were extracted from Ladino clover than from birdsfoot trefoil.

Vaginal cell changes were found in ewes on Ladino clover and birdsfoot trefoil which were characteristic of those that normally developed following the appearance of high levels of both natural and synthetic estrogens in the body. These findings supported the supposition and results of Engle that an estrogen-like substance was present in Ladino clover and birdsfoot trefoil. This material may be the factor that caused delayed conception and extended lambing seasons.

Summary

Vaginal smears, from 8 virgin, yearling, Columbia ewes, stained by the Papanicolaou method were studied.

Anestrous smears, from normal control ewes on dry feed, showed only scattered normal, basophilic, epithelial cells. A similar smear was found during proestrus and estrus from control ewes during a normal estrous cycle in September and October.

After estrogens were administered to the anestrous control ewes, smears became thick and cheesy and were composed of masses of cornified cells and some leukocytes. Only a few basophilic cells were present. These smears were similar to the smears taken from the control ewes during the metestrous and diestrous phases of the normal estrous cycle.

Anestrous smears from ewes on Ladino clover and birdsfoot trefoil were thick and cheesy; nearly all cells were cornified, and many leukocytes and bacteria were present. These smears were similar to the smears from the estrogen-treated anestrous control ewes, and the metestrous and diestrous smears from the control ewes during a normal estrous cycle. It is suggested that the extensive cornification and desquamation of vaginal epithelium observed in anestrous ewes was caused by the presence of an estrogen-like substance in Ladino clover and birdsfoot trefoil pastures.

This is probably the substance that interfered with conception during the fall breeding season, as previously reported.

Addendum

Since the work here reported was completed, the Western Utilization Research and Development Division, United States Agricultural Research Service, Albany, Calif., has announced the purification and crystallization of a highly active estrogen from Ladino clover which they have named "Coumestrol." *

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The Influence of Season on Blood Plasma Cholesterol in the Ewe

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IT IS WELL KNOWN that there is a relationship between thyroid function and blood cholesterol levels. It has been shown by Schmidt and Hughes,[13] Thompson and Long,[14] Chaikoff *et al.*,[5] and others that thyroidectomy of the dog is followed by an increase in blood cholesterol. In man, partial thyroidectomy is followed by hypercholesterolemia.[10] Wilkins *et al.*[15] and others have repeatedly shown that there is a relationship between hypothyroidism and hypercholesterolemia in children, although some hypothyroid individuals do not exhibit increased blood cholesterol. Barrick *et al.*[1] found that hypothyroidism of sheep, produced by the administration of thiouracil and thiourea, was accompanied by an increase in plasma cholesterol of approximately 30 to 40 per cent.

In rats and mice, Dempsey and Astwood,[6] Hurst and Turner,[9] and others have repeatedly demonstrated that the thyroid secretion rate is increased when the environmental temperature is lowered beyond the range of thermoneutraltiy. Several investigators have associated lowered fertility and hypothyroidism in sheep[1, 8] and in cattle.[4, 11]

Henneman *et al.*,[8] using a technique involving the administration of I^{131} and l-thyroxine, found that, in female sheep, the daily thyroxine secretion rate was minimal in July and highest in winter and early spring. Lactating ewes had a higher thyroxine-secretion level than either pregnant or nonpregnant mature ewes, but there appeared to be no difference between pregnant and nonpregnant ewes.

Because of the seasonal breeding pattern of sheep and the seasonal differences in thyroid function of sheep, it was decided to study the relationship of season to blood plasma cholesterol levels of breeding ewes.

MATERIALS AND METHODS

A total of 627 blood plasma cholesterol determinations were made on 157 sheep during a two-year period. Plasma cholesterol was determined by a slight modification of the method of Pjoan and Walter.[12] The cholesterol in 0.2 ml. of plasma was extracted twice by a mixture of 3 parts of 95 per cent ethanol and 1 part anhydrous ether, evaporated to dryness in a water bath, and the dry residue taken up in 10 ml. of chloroform and placed in a water bath at 25 C. To the chloroform solution was added 4 ml. of acetic anhydride and 0.1 ml. of concentrated sulfuric acid. The mixture was thoroughly stirred with a footed glass rod and readings were taken in an Evelyn photoelectric colorimeter at 15, 20, and 25 minutes, or until maximum color had developed. Cholesterol values were obtained from standards prepared from pure cholesterol.

With the exception of 5 animals, the ewes were of the western type and appeared to be of predominantly Hampshire, Suffolk, and Rambouillet breeding. Each ewe was fed approximately 1 lb. daily of a mixture of shelled yellow corn, whole oats, and pelleted Purdue cattle supplement A. Second-cutting alfalfa hay was fed ad libitum and a mineral mixture containing iodized salt, calcium, and phosphorus was available at all times. In all experiments, the control ewes were kept in a three-sided shed which was open on the south side. These animals were subjected to natural light and temperature conditions at all times.

In some experiments, ewes were kept in stalls (12 by 13 ft.) which were insulated from light. Light was provided by one 200-watt incandescent bulb in each stall. The maximum light at eye level of the sheep was 9 foot-candles in the center of the stall. The stalls were either ventilated with exhaust fans or cooled with two standard room-type air conditioners, each with 1 ton refrigeration capacity. Humidity was not controlled. More specific environmental conditions will be described for each experiment.

The data were analyzed by appropriate statistical procedures, principally the analysis of variance.

METHODS AND RESULTS

Trial 1.—The control ewes (group 1) were kept in a normal environment from March 22 until July 12. The ewes in groups 2 and 3 were exposed to ten hours of natural daylight and 14 hours of complete darkness by confinement in a stall each day. In addition, group 3 ewes were treated daily with 15 mg. of progesterone

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TABLE 1—The Influence of Season and Light on the Blood Plasma Cholesterol of Ewes—Trial 1

Blood samples taken	Av. plasma cholesterol (mg./100 ml.)			
	Group 1—9 ewes controls, normal environment	Group 2—9 ewes 10 hr. daylight, 14 hr. darkness, per 24 hr.	Group 3—10 ewes 10 hr. darkness, 10 hr. daylight, plus progesterone	Group 4—10 ewes 2 daylight, 2 dark periods per 24 hr.
March 22	54.4	55.2	58.7	57.9
April 26	53.3	51.6	54.8	58.2
May 24	42.2	45.3	48.2	49.5
July 5	51.9	51.0	55.1	53.2

on April 26, 27, and 28. The ewes in group 4 were subjected to two long dark periods and two short light periods in each 24 hours. These animals were confined in a dark stall from 7:00 a.m. until 5:00 p.m. They remained outside for the remainder of each day from March 22 until July 12.

The seasonal changes in blood plasma cholesterol are summarized (table 1).

The data were analyzed by the method of analysis of variance using a nested factorial design. It was shown that the mean group values were an unbiased estimate of the average for each group, and the group averages were, therefore, used as the experimental unit in the analysis. There were no significant differences between treatments but a highly significant ($P < 0.01$) difference between days. A few days prior to the blood sampling on May 22, the night temperature dropped to 40 to 26 F. for several nights. While this is relatively cold for May and may have produced the consistent reduction in cholesterol observed in all groups, it does not explain the failure of much lower winter temperature to produce similar effects in other trials.

Trial 2.—The control ewes were exposed to the normal fluctuating environment of an open shed from July 15 to February 22. The ewes in group 2 were confined to stalls throughout the same period and exposed to ten hours of artificial light and 14 hours of darkness each day. From July 15 until the beginning of October, artificial cooling was provided for group 2. The equipment was not designed for constant temperature maintenance and the temperature fluctuated between 70 and 80 F. However, the temperatures in the stalls were consistently lower than the temperatures of the summer environment of the control ewes.

The data are summarized (table 2). As in the previous trial, there were highly significant differences between days ($P < 0.01$) but no significant treatment differences. The lowest cholesterol values for both groups occurred in September; the values for July and October were virtually identical. Most of the ewes had become pregnant at the time of the October 20 blood sampling. However, comparisons of July and October cholesterol values clearly indicate that differences in environmental temperature, day length, or reproductive status did not affect circulating cholesterol levels.

Trial 3.—The control ewes were maintained in an open shed during the trial, from March 15 until July 15. The ewes in group 2 were exposed to ten hours of daylight and 14 hours of darkness each day by confinement in darkened stalls from 5:00 p.m. until 7:00 a.m. During June and July, the stalls were mechanically cooled during the night, with temperatures ranging from 70 to 80 F.

TABLE 2—The Influence of Season, Light, and Pregnancy on Blood Plasma Cholesterol—Trial 2

Blood samples taken	Av. plasma cholesterol (mg./100 ml.)	
	Group 1—20 ewes controls, normal environment	Group 2—20 ewes 10 hr. artif. light, 14 hr. darkness/day; artificial cooling until Oct. 1
July 15	53.7	53.4
Sept. 11	47.1	51.1
Oct. 20 *	53.7	54.1
Dec. 21	58.6	65.6
Feb. 22	56.8	54.4

* Most of the ewes were pregnant by October 20.

TABLE 3—The Influence of Season and Light on Blood Plasma Cholesterol—Trial 3

Blood samples taken	Av. plasma cholesterol (mg./100 ml.)	
	Group 1—19 ewes controls, normal environment	Group 2—20 ewes 10 hr. daylight, 14 hr. darkness each day
March 24	58.10	55.7
April 29	50.20	49.6
June 6	54.10	54.7
July 8	55.05	54.3

Although there is a highly significant ($P < 0.01$) difference between days in cholesterol values, there is no difference between treatments (table 3). It is obvious that the normal seasonal increases in light and temperature to which the control ewes were exposed did not produce a difference between March and July cholesterol levels.

Trial 4.—During this trial, July 15 to October 7, the control ewes were exposed to a normal environment. The sheep in group 2 were kept in an air-conditioned stall throughout this period and were exposed to ten hours of artificial light each day. The temperatures ranged between 75 to 80 F. In the control group, the maximum daily temperature usually exceeded 85 F. and the minimum temperature was seldom less than 65 F.

An analysis of variance showed that there was a highly significant ($P < 0.01$) difference (table 4) between days in the control group, in which there appeared to be a linear relationship between month and blood plasma cholesterol.

TABLE 4—The Influence of Season and Light on Blood Plasma Cholesterol—Trial 4

Blood samples taken	Av. plasma cholesterol (mg./100 ml.)	
	Group 1—20 ewes controls, normal environment	Group 2—20 ewes 10 hr. artif. light 14 hr. darkness and artificial cooling
Aug. 6	53.3	58.5
Sept. 6	59.3	58.5
Oct. 4	63.3	58.8

DISCUSSION

While it is logical to expect a reduction in thyroid activity and an accompanying increase in plasma cholesterol during the summer months, an examination of all the data does not justify this conclusion. The highest mean cholesterol level (65.6 mg./100 ml.) for a group of 20 ewes in early pregnancy was obtained in trial 2 on December 21. Mean values of 58 mg./100 ml. of blood cholesterol for other groups of ewes have been observed in March, April, August, October, and December. It can not be concluded from these data, therefore, that any significant alteration in blood plasma cholesterol of sexually mature ewes has been brought about by normal seasonal changes, controlled lighting, partial temperature control, or reproductive state.

Barrick *et al.*,[1] in experiments with western feeder lambs, showed that hypothyroidism produced by the oral administration of thiouracil or thiourea significantly increased blood plasma cholesterol by approximately 30 to 40 per cent. Observations on normal fattening lambs in winter and summer experiments did not indicate any seasonal effects. In one summer trial, the mean cholesterol value over a ten-week period was 57 mg./100 ml. in contrast to a winter feeding experiment in which the mean plasma cholesterol was 60 mg./100 ml.

It has been hypothesized that there might be seasonal differences in blood plasma cholesterol levels of sexually mature female sheep, which could be associated with reproductive status. Brody and Procter[4] observed seasonal differences in metabolic rate which they attributed to the seasonal reproductive cycle. Berliner and Warbritton[2] and Bogart and Mayer,[3] in studies of the cause of seasonal changes in fertility in the ram, suggested that the thyroid gland was one of the mechanisms involved. More recently, Henneman *et al.*[8] reported a great reduction in the thyroid secretion rate of breeding ewes in July. However, in nonlactating open or pregnant ewes, the differences in daily l-thyroxine secretion were relatively small in September and December by contrast with the July values.

Since the metabolism of cholesterol is related to many factors other than thyroid function, it would not be expected to be as sensitive an indicator of thyroid secretion as the estimation of thyroxine secretion.

The values obtained in this study are closely comparable with the mean cholesterol value for ewes and lambs reported by Hackett *et al.*[7] (64.6 ± 3.3 mg./100 ml.).

SUMMARY AND CONCLUSIONS

A total of 627 blood plasma cholesterol determinations were made on 157 sexually mature ewes during a two-year period.

There were no significant seasonal differences in the blood plasma cholesterol levels of ewes which were kept in a normal fluctuating seasonal environment.

The alteration of day length and the modification of the normal summer en-

vironment by artificial cooling had no effect on plasma cholesterol levels.

There were no significant differences in the blood plasma cholesterol levels of pregnant and nonpregnant ewes.

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Nematocidal Activity of Some Organic Phosphorus Compounds Against Horse Strongyle Larvae *in Vitro*

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THE RECENT introduction of organic phosphorus insecticides and the large amount of research associated with their development have made many of these compounds available for experimental research. In preliminary screening tests of eight organic phosphorus compounds, Levine *et al.*[11] found that some of them were effective against horse strongyle larvae *in vitro*. The results were so encouraging that further study was believed justified, and this paper reports the results obtained with 173 organic phosphorus compounds.

MATERIALS AND METHODS

The screening technique used in this study has been described by Levine.[8,9] Briefly, the compound to be tested was mixed with talcum powder to give it body; this mixture was in turn mixed with nine times its weight of fresh horse feces containing strongyle eggs. The resulting mixture was suspended in a cheesecloth bag in a loosely covered, wide-mouthed jar and incubated in a low-temperature incubator at 20 C. for a week. At the end of this period, the culture was examined for larvae by the Baermann technique. The cheesecloth bag was placed in a funnel provided with a short length of rubber tubing closed by a pinch clamp. The culture jar was rinsed with warm water, and the rinse water plus additional warm water was poured over the bag to about half its height in the funnel. The preparation was allowed to stand for at least three hours, to allow living larvae to migrate out of the feces and fall down into the column of water in the funnel stem. The liquid from the funnel was collected in a Petri dish and examined under a microscope for larvae. Untreated controls, both with and without talcum powder, were set up and examined with each run. Each compound was tested at concentrations of 0.01, 0.005, and 0.0025, and 0.001 M. Lower concentrations were tested later if the compound was found to kill all larvae at these concentrations.

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RESULTS

One or more derivatives of 26 types of organic phosphorus compounds were screened for nematocidal activity against horse strongyle larvae *in vitro* (table 1). Of the 173 compounds tested, 104 (60.2%) were inactive at a concentration of 0.01 M. Larvae were killed or prevented from developing in 25 (14.5%) at concentrations of 0.01 M; in 17 (9.8%) at 0.005 M; in 16 (9.3%) at 0.0025 M; in 3 (1.7%) at 0.001 M; in 2 (1.2%) at 0.0005 M; in 3 (1.7%) at 0.00025 M; and in 3 (1.7%) at 0.00005 M. The last three compounds were the most active tested in this laboratory.

The lowest effective concentrations of the 69 compounds * which showed activity against horse strongyle larvae *in vitro* are given (table 2).

DISCUSSION

The insecticidal action of the organic phosphorus compounds is generally believed to be due to direct inhibition of the action of various insect esterases, of which the most important is acetylcholine esterase (Metcalf,[13] Kearns [6]). Little is known about choline esterases in nematodes. Von Brand [14] discussed the question and concluded that there was some doubt as to whether acetylcholine esterase occurs in nematodes, although he reported that Bueding had found it in filariae and in ascarid muscle. As a consequence, most of the inferences concerning the action of organic phosphorus compounds on nematodes must be drawn from the information available for insects and mammals. However, the presence of a choline esterase may not be necessary to explain the action of these compounds on nematodes, since the compounds can inactivate other enzymes, including chymotrypsin and phenyl acetate esters.[12]

* The names of the ineffective compounds are omitted to conserve space, but can be obtained from the authors on request.

The compounds most active against horse strongyle larvae were the diethyl and dipropyl esters of 2,2-dichlorovinyl-phosphoric acid and the diethyl ester of 4-oxopenten-1-yl phosphonic acid (Shell OS 1808). The first two compounds are of particular interest because they are the diethyl and dipropyl homologs of the active insecticide, DDVP,* which is a dimethyl ester. It has been speculated (Babers and Mitlin,[2] Casida,[4] Arthur and Casida [1]) that the insecticide, Dipterex (the dimethyl ester of 2,2,2-trichloro-1-hydroxyethyl phosphoric acid), is converted in the insect to DDVP by dehydrochlorination and rearrangement, and that it is the latter compound which actually kills the insect. Arthur and Casida,[1] however, have not been able to demonstrate this conversion *in vivo*. Babers and Mitlin [2] found that DDVP was 64 times as active as Dipterex against houseflies, and that the diethyl and dipropyl homologs of DDVP were, respectively, 12.7 and 1.5 times as active as Dipterex. Against cockroach cholinesterase, the differences were even greater; DDVP and its diethyl and dipropyl homologs were, respectively, 64.0, 120.0, and 35.2 times as active as Dipterex.

Our results indicate that there is no necessary relationship between insecticidal and nematocidal activity, for DDVP was found to be only one tenth as active against horse strongyle larvae as Dipterex, while the diethyl and dipropyl homologs of DDVP were five times as active as Dipterex, and

* *See* table 2 for sources and manufacturers of compounds.

TABLE 1—Activity of Organic Phosphorus Compounds Against Horse Strongyle Larve in Vitro

Derivatives of organic phosphorus compounds	No. of compounds									
		Lowest lethal concentration (M)								
	Inactive at 0.01 M	0.01	0.005	0.0025	0.001	0.0005	0.00025	0.0001	0.00005	Total
PENTAVALENT PHOSPHORUS										
Phosphoric acid	22	6	1	2	3	1	1	0	2	38
Phosphorothionic acid	12	3	2	2	0	0	0	0	0	19
Phosphorothiolothionic acid	6	2	1	1	0	0	0	0	0	10
Phosphorodithiolothionic acid	0	0	0	1	0	0	0	0	0	1
Phosphonic acid	23	5	3	6	0	0	1	0	1	39
Phosphinic acid	2	0	0	0	0	0	0	0	0	2
Phosphoramidothionic acid	0	0	2	1*	0	0	0	0	0	3
Phosphorochloridic acid	2	0	0	0	0	0	0	0	0	2
Phosphorochloridothionic acid	0	0	0	1	0	0	0	0	0	1
Phosphorodichloridic acid	1	0	0	0	0	0	0	0	0	1
Phosphonium	1	1	0	0	0	1	0	0	0	3
Pyrophosphoric acid	3	0	0	0	0	0	0	0	0	3
Pyrophosphorodithionic acid	1	1	0	0	0	0	0	0	0	2
Pyrophosphorodiamidic acid	1	0	0	0	0	0	0	0	0	1
Trithiopyrophosphoric acid	1	0	0	1	0	0	0	0	0	2
α, ω-alkyldiphosphonic acid	5	0	0	0	0	0	0	0	0	5
Diamidochlorophosphine oxide	0	0	1	0	0	0	0	0	0	1
Amidodichlorophosphine oxide	1	0	0	0	0	0	0	0	0	1
TRIVALENT PHOSPHORUS										
Phosphorous acid	11	3	3	1	0	0	0	0	0	18
Phosphorous acid complexes	1	2	2	0	0	0	0	0	0	5
Phosphorotrithious acid	3	1	1	0	0	0	1	0	0	6
Phosphonous acid	1	0	0	0	0	0	0	0	0	1
Phosphine	1	0	0	0	0	0	0	0	0	1
Phosphorochloridous acid	2	0	1	0	0	0	0	0	0	3
Phosphorodichloridous acid	2	1	0	0	0	0	0	0	0	3
HEPTAVALENT PHOSPHORUS										
Fluorophosphate	2	0	0	0	0	0	0	0	0	2
TOTAL	104	25	17	16	3	2	3	0	3	173

* The actual readings in several tests were 0.0005 to 0.005 M.

TABLE 2—Compounds that Killed or Prevented Development of All Horse Strongyle Larvae in Vitro in the Concentration Indicated*

Compound	Source**	Lowest active concentration (Molar)	Lowest active concentration (%)
PHOSPHORIC ACID DERIVATIVES			
0-(3,5,5-trimethylhexyl)-	1	0.01*	0.22*
0,0-bis(2-ethylhexyl)-	1	0.01	0.32
0-(2,2-dichlorovinyl)-, 0,0-diethyl-	3	0.001†	0.019†
0-(2,2-dichlorovinyl)-, 0,0-dimethyl- (DDVP)	3, 4	0.0025†	0.055†
0-(2,2-dichlorovinyl)-, 0,0-dipropyl-	3	0.00005†	0.00125†
0-(2-chlorovinyl)-, 0,0-dipropyl-	3	0.001†	0.024†
0-(2,2-dichlorovinyl)-, 0,0-dipropyl-	3	0.00005†	0.0014†
0-(2-chlorovinyl)-, 0,0-diisopropyl-	3	0.01†	0.24†
0-(2,2-dichlorovinyl)-, 0,0-diisopropyl-	3	0.001†	0.028†
0-(2-chlorophenyl)-, 0,0-diphenyl-	3	0.0025†	0.068†
0-(2,2-dichlorovinyl)-, 0,0-dibutyl-	3	0.00025†	0.008†
0,0,0-triallyl-	1	0.005†	0.095†
0,0,0-tributyl-	1	0.01†	0.27†
0,0,0-triisobutyl-	1	0.01†	0.27†
0-(2,4,5-trichlorophenyl)-, 0,0-diethyl-	2	0.0005†	0.0165†
0-(2-chlorophenyl)-, 0,0-diphenyl-	1	0.01†	0.36†
PHOSPHOROTHIONIC ACID DERIVATIVES			
0-(2-mercaptoethyl)-, 0,0-diethyl-	2	0.005	0.115
0-(2-chloro-4-nitrophenyl)-, 0,0-diethyl-	2	0.01†	0.32†
0-(3-chloro-4-nitrophenyl)-, 0,0-diethyl- (Chlorthion)	5	0.0019	0.06
0-(*x*-chloro-4-nitrophenyl)-, 0,0-diethyl-	5	0.01‡	0.33‡
0-(*p*-phenyldiazophenyl)-, 0,0-diethyl-	2	0.01†	0.35†
0-[2-isopropyl-4-methylpyrimidyl(6)]-, 0,0-diethyl- (Diazinon)	5	0.0025	0.08
0-(3-chloro-4-methylumbelliforone)-, 0,0-diethyl- (Bayer 21/199)	5	0.0074	0.27
PHOSPHORAMIDOTHIONIC ACID DERIVATIVES			
0,0-bis-(6-*tert*-butyl-*m*-tolyl)-	1	0.01†	0.42†
0,0-dimethyl-, diester with diethyl mercaptosuccinate (Malathion)	5	0.005	0.02
S-(2-ethylmercaptoethyl)-, 0,0-dimethyl-	6	0.0025†	0.062†
S-(2-ethylmercaptoethyl)-, 0,0-diethyl-	6	0.01†	0.27†
PHOSPHORODITHIOLOTHIONIC ACID DERIVATIVE			
0-(2-chlorovinyl)-, S,S,-diethyl-	3	0.0025†	0.065†
PHOSPHONIC ACID DERIVATIVES			
m-bromo phenyl-	1	0.01†	0.24†
2,2,2-trichloro-1-hydroxyethyl-, 0,0-dimethyl- (Bayer L13/59, Dipterex)	7	0.0001	0.004
		0.00025	0.007
2,2-dichloro-1-hydroxyethyl-, 0,0-dimethyl-, acetate	3	0.0025†	0.065†
2,2,2-trichloro-1-hydroxyethyl-, 0,0-dimethyl-, acetate	3	0.0025†	0.075†
2,2,2-trichloro-1-hydroxyethyl-, 0,0-dimethyl-, octanoate	3	0.0037†	0.14†
2,2-dichloro-1-hydroxyethyl-, 0,0-diethyl-	3	0.0025	0.062
2-chlorovinyl-, 0,0-diethyl-	8	0.005	0.1
4-oxopenten-1-yl-, 0,0-diethyl- (OS 1808)	8	0.00005	0.0011
2-ethylhexyl-, 0,0-diethyl-	2	0.005†	0.125†
mercury acetate, diethoxyphosphinyl-	1	0.01	0.40
butyl-, 0,0-dibutyl-	1	0.0025	0.063
1-hydroxy-1-methyl-2-carbethoxy-ethyl-, 0,0-bis-(*p*-chlorobenzyl)-	3	0.01	0.46
2,2-dichloro-1-hydroxyethyl-, 0,0-bis-(*p*-chlorobenzyl)-	3	0.0025	0.11
2,2,2-trichloro-1-hydroxyethyl-, 0,0-bis-(*p*-chlorobenzyl)-	3	0.0025	0.12
1-hydroxypropyl-, 0,0-bis-(*p*-chlorobenzyl)-	3	0.01	0.39
1-hydroxy-1-methylpropyl-, 0,0-bis-(*p*-chlorobenzyl)-	3	0.01†	0.40†
PHOSPHORAMIDOTHIONIC ACID DERIVATIVES			
N-carbethoxymethyl-, 0,0-diethyl-	3	0.005†	0.13†
N-(2-carbethoxyethyl)-, 0,0-diethyl-	3	0.005†	0.14†
0-methyl-, 0-(2,4,5-trichlorophenyl)- (Dow ET-15)	9	0.00078	0.03
		0.005	0.16
PHOSPHOROCHLORIDOTHIONIC ACID DERIVATIVE			
0,0-dibutyl-	1	0.005	0.12
PHOSPHONIUM DERIVATIVES			
Phosphonium bromide, triphenylisopropyl-	10	0.008	0.31
Phosphonium iodide, triphenylmethyl-	10	0.0005	0.025
PYROPHOSPHORODITHIONIC ACID DERIVATIVE			
bis-(0,0-diethyl)-	8	0.005†	0.16†
TRITHIOPYROPHOSPHORIC ACID DERIVATIVE			
bis-[0,0-(*x*-isopropyl-*m*-toloxy)]-	1	0.0025†	0.208†
DIAMIDOCHLOROPHOSPHINE OXIDE DERIVATIVE			
N,N¹-diphenyl-	1	0.005	0.135
PHOSPHOROUS ACID DERIVATIVES			
0,0-[bis-(3,5,5-trimethylhexyl)]-	1	0.005	0.165
0,0-[bis-(2-ethylhexyl)]-	1	0.01	0.31
0,0,0-[tris-(2-chloroethyl)]-	2	0.005†	0.135†
0,0,0-tris-(2-ethylhexyl)-	1	0.01	0.42
0,0,0-tris-(methylheptyl)-	1	0.005	0.21
0,0,0-trioctyl- (mixture of isomers)	1	0.01†	0.42†
0-(2,4-dichlorophenyl)-, 0,0-bis-(2-chloroethyl)-	2	0.0025†	0.088†

TABLE 2 (Continued)—Compounds that Killed or Prevented Development of All Horse Strongyle Larvae in Vitro in the Concentration Indicated*

Compound	Source**	Lowest active concentration (Molar)	Lowest active concentration (%)
PHOSPHOROUS ACID COMPLEXES			
0,0,0-tris-(2-ethylhexyl)-, complex with 1 f. wt. zinc chloride	1	0.01†	0.55†
0,0,0-tris-(2-ethylhexyl)-, complex with 1 f. wt. tin (II) chloride	1	0.01†	0.61†
0,0,0-tris-(2-ethylhexyl)-, complex with 1 f. wt. mercury (II) chloride	1	0.005†	0.345†
0,0,0-tris-(2-ethylhexyl)-, complex with 1 f. wt. mercury (II) bromide	1	0.005	0.39
PHOSPHOROTRITHIOUS ACID DERIVATIVES			
0,0,0-trimethyl-	2	0.01	0.17
0,0,0-triethyl-	2	0.005	0.105
0,0,0-tris-(*n*-propyl)-	2	0.00025†	0.0065†
PHOSPHOROCHLORIDOUS ACID DERIVATIVE			
0,0,-bis-(*o*-tolyl)-	2	0.005	0.145
PHOSPHORODICHLORIDOUS ACID DERIVATIVE			
0 phenyl-	2	0.01	0.20

* Including organic phosphorus compounds tested and reported earlier;[3,4,5] † some eggs hatched but only dead larvae found; ‡ no lower concentrations tested.

** The compounds were received from the following sources: (1) National Research Council, Chemical-Biological Coordination Center, Washington, D.C.; (2) L. E. Goyette, Virginia-Carolina Chemical Corp., Richmond, Va.; (3) C. N. Smith, Entomology Research Branch, U.S.D.A., Orlando, Fla.; (4) P. Rothberg, Montrose Chemical Corp., Newark, N.J.; (5) W. N. Bruce, Illinois Natural History Survey, Urbana; (6) R. J. Geary, Geary Research Laboratories, Blue Point, L.I., N.Y.; (7) R. von Rümker, Chemagro Corp., New York, N.Y.; (8) C. W. Kearns, Department of Entomology, University of Illinois, Urbana; (9) J. E. Johnson, Dow Chemical Co., Midland, Mich.; (10) C. S. Marvel, Department of Chemistry, University of Illinois, Urbana; (11) D. L. Shankland, Stauffer Chemical Corp., Omaha, Neb.; (12) G. F. Otto, Abbott Laboratories, North Chicago, Ill.

the dibutyl homolog of DDVP had the same activity as Dipterex.

This relationship also is interesting because it illustrates the effect on activity of increasing molecular size. It is likely that nematocidal activity would decrease progressively in the higher homologs of DDVP, but these were not available for testing.

The series of phosphorotrithious acid derivatives again illustrates the effect of molecular size on activity. Trimethyl phosphorothioite, the lowest member of the series, was active at 0.01 M, while the triethyl and tri-*n*-propyl esters were active at 0.005 M and 0.00025 M, respectively. Activity then dropped abruptly, the tributyl, tripentyl, and trioctyl esters being inactive at 0.01 M.

Two other compounds are of particular interest. One, the diethyl ester of 2,4,5-trichlorophenyl phosphoric acid, killed all larvae at a concentration of 0.0005 M, while its symmetrical isomer, the diethyl ester of 2,4,6-trichlorophenyl phosphoric acid, was inactive at 0.01 M, the highest concentration tested. The stereochemical relationship between the two compounds is striking, and these findings may be a clue to the mode of action of some of these organic phosphorus compounds. Their activity is considered due to competition with acetylcholine and related compounds for cholinesterases. All three types of compound have two sites of union, one positively and the other negatively charged (Kearns[6]). The positively charged site of acetylcholine reacts with the negatively charged site of cholinesterase, and the negatively charged site of the former with the positively charged site of the latter. Cholinesterase antagonists act like acetylcholine.

In the organic phosphates considered here, these sites are the oxo- group attached to the phosphorus atom and one of the chlorines attached to the phenyl group. There are three of these chlorine atoms. They are attracted to the oxygen, and their distances from it and the consequent configuration of the molecule result from the interaction of the forces involved. These distances differ for the 2,4,5- and 2,4,6-trichlorophenyl compounds. In order to react with the compounds which they antagonize, the distances between the two sites must be the same on both molecules or the union will not be firm. This is apparently the case with the 2,4,5- but not with the 2,4,6- compound.

The asymmetrical 2,4,5-trichlorophenyl configuration was present in three additional compounds. Of these, 0-methyl, 0-2,4,5-trichlorophenyl phosphoramidothionate (Dow ET-15) proved to be highly active, killing all horse strongyle larvae at 0.00078 M (0.03%) and 0.005 M (0.16%) in two tests. However, the dimethyl ester (Dow ET-14, ET-57) and the diethyl ester of 2,4,5-trichlorophenyl phosphorothionic acid were inactive at 0.1 M (2.5%) and 0.01 M (0.35%), respectively. This is of particular interest, since ET-57 has received much attention recently because of its insecticidal activity and has also been shown by Herlich and Johnson [5] to be highly active against *Haemonchus contortus* (but much less so against other nematodes) in cattle.

It is worthwhile to compare the 2,4,5-trichlorophenyl derivatives of phosphoric and phosphorothionic acids with DDVP and Dipterex. It is generally believed that phosphorothionates are converted to phosphates before they can act as insecticides.[6] This also could be true for nematodes, since both Dipterex (a phosphonate) and DDVP (the phosphate into which it has been suggested Dipterex is changed) are nematocidal. However, the 2,4,5-trichlorophenyl phosphate is highly nematocidal, while its thiono- analog is inactive. Perhaps these nematodes do not have the enzymes necessary to make the conversion to the more active compound. The activity of the phosphoramidothionic analog (Dow ET-15) may be explained by the presence of the amido group. The study of related compounds might prove worthwhile.

Further studies probably will reveal several additional nematocidal compounds, some of which may be satisfactory anthelmintics. In our screening tests of miscellaneous compounds, few were found to have nematocidal activity, but 40 per cent of the organic phosphorus compounds screened showed some activity. Several thousand organic phosphorus compounds have been synthesized, and there is every reason to believe that among them will be a number which combine high anthelmintic activity with low toxicity for the host.

Summary

Screening tests for nematocidal activity against horse strongyle larvae *in vitro* were carried out on 173 organic phosphorus compounds of 26 types. Of these, 39.8 per cent showed some activity at a concentration of 0.01 M or lower. The most active compounds and the concentrations at which they killed or prevented the development of all larvae were 0-(2-chlorovinyl)-0,0-dimethyl phosphate (0.001 M); 0-(2-chlorovinyl)-0,0-dipropyl phosphate (0.0001 M); 0-(2,2-dichlorovinyl)-0,0-diethyl phosphate (0.00005 M); 0-(2,2-dichlorovinyl)-0,0-dipropyl phosphate (0.00005 M); 0-(2,2-dichlorovinyl)-0,0-diisopropyl phosphate (0.001 M); 0-(2,2-dichlorovinyl)-0,0-dibutyl phosphate (0.00025 M); 0-(2,4,5-trichlorophenyl)-0,0-diethyl phosphate (0.0005 M); 2,2,2-trichloro-1-hydroxyethyl-0,0-dimethyl phosphonate (Dipterex) (0.0001 to 0.00025 M); 4-oxo-penten-1-yl-0,0-diethyl phosphonate (OS 1808) (0.00005 M); triphenylmethyl phosphonium iodide (0.0005 M), and 0,0,0-tri-(*n*-propyl) phosphorotrithioite (0.00025 M).

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Histological Aspects of *Klossiella Equi* in the Kidney of a Zebra

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PROTOZOAN PARASITES of the genus Klossiella have been reported from the kidneys of the mouse, the guinea pig, the jerboa, the American jack and the horse.[1-7] The purpose of this article is to present a histological description of Klossiella in the kidney of an African zebra. To preserve continuity, the descriptive terms * proposed by Seibold and Thorson [4] are used wherever applicable.

The host of the parasite was a zebra of unknown age, which had arrived in the United States from Africa six months prior to death and was housed in a zoo in Miami, Fla. Death was attributed to acute indigestion. At necropsy, there was no specific gross evidence of any morbid condition, other than slight gastroenteritis.

HISTOPATHOLOGICAL FINDINGS

Routine microscopic examination of the major visceral organs and brain revealed no significant alterations except the sporogonic cycle of a protozoan parasite in the kidneys. Critical study of the other organs failed to reveal any suggestion of parasitic forms.

In the kidneys, the parasites were located within the lining epithelial cells in the tubules of the thick limb of Henle's loops, situated in the renal medullary rays. The degree of parasitism was extremely heavy. The parasitized epithelial cells tended to protrude into and partially occlude the lumens of the affected tubules, which showed varying degrees of dilatation, depending upon the number and size of parasites present. Specific host-tissue reaction was not evident, but in many of the distal collecting tubules there were variable amounts of eosinophilic amorphous debris, presumably degenerated material eliminated from the parasitized areas.

DESCRIPTION OF THE PARASITE

The heavy degree of parasitism made it possible to follow gradations in the developmental stages of the sporogonic cycle of the parasite. The smallest form observed (fig. 1A) was located within a cytoplasmic vacuole of the enlarged renal epithelial host cell. The vacuole was apparently due to shrinkage from fixing, dehydrating, and embedding of the tissue. This form was approximately 7 by 9 μ in size, and consisted of a uninuclear, round or oval mass of reticulated protoplasm. The single small nucleus, which contained basophilic chromatin particles, was located at the periphery of the mass. In a majority of the organisms, a distinct delimiting nuclear membrane was present. In addition to the uninuclear mass, approximately 25 per cent of these forms showed one to four spherical or signet-ring-shaped, eosinophilic masses, approximately 1 μ in diameter, lying free in the vacuole (fig. 1B). Each of these small masses contained one or more basophilic chromatin particles. Occasionally, one of these small masses was seen in contact with the main uninuclear form.

As the uninuclear form increased in size, chromatin particles within the protoplasmic mass appeared to migrate toward its periphery. When the form had attained an approximate size of 16 by 20 μ (fig. 2), it had developed into a round or oval mass of reticulated protoplasm, partially circumscribed, with multiple nuclei arranged along the border. These nuclei were vaguely defined areas of condensed protoplasm containing basophilic granules. As the organism continued to grow, it showed wavelike projections of the protoplasm at the periphery. At 25 to 35 μ these projections had developed into a contiguous layer of small buds along the periphery, each

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* "Uninuclear form," "multinuclear form," "budding form," and "oocyst with spores," are terms denoting four principal stages in the development of the oocyst.

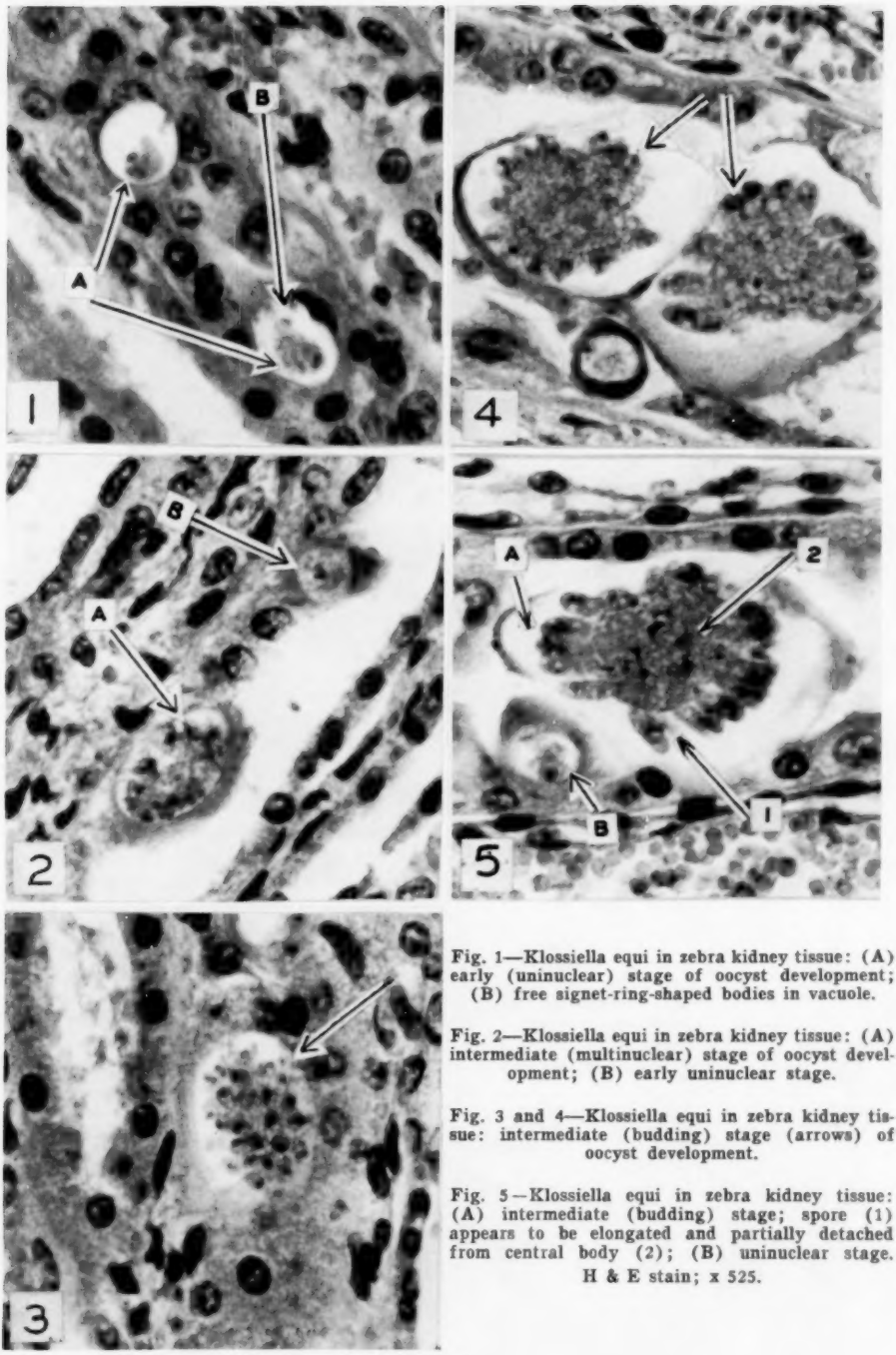

Fig. 1—Klossiella equi in zebra kidney tissue: (A) early (uninuclear) stage of oocyst development; (B) free signet-ring-shaped bodies in vacuole.

Fig. 2—Klossiella equi in zebra kidney tissue: (A) intermediate (multinuclear) stage of oocyst development; (B) early uninuclear stage.

Fig. 3 and 4—Klossiella equi in zebra kidney tissue: intermediate (budding) stage (arrows) of oocyst development.

Fig. 5—Klossiella equi in zebra kidney tissue: (A) intermediate (budding) stage; spore (1) appears to be elongated and partially detached from central body (2); (B) uninuclear stage. H & E stain; x 525.

containing two small, basophilic chromatin granules (fig. 3). Typical budding forms (fig. 4) measured 35 to 45 μ and consisted of large, oval, centrally located masses of reticulated protoplasm surrounded by buds. Repeated observations on the various stages of development of these budding forms (figs. 3 to 5) gave the impression that the buds ultimately lengthened and were cast off from the central mass to become spores. The central mass of protoplasm then apparently disappeared.

A final stage, the mature oocyst, consisted of a group of spores enclosed in a thin-walled sac, apparently the remnant of the parasitized host cell. This form (fig. 6) varied in size from 50 by 30 μ to 95 by 30 μ. The size was somewhat dependent upon the level at which the parasite was sectioned. The individual spores were ovoid and measured 4 by 6 to 6 by 10 μ. The younger spores showed an affinity for eosin dyes, while the more mature spores showed an affinity for basophilic dyes.

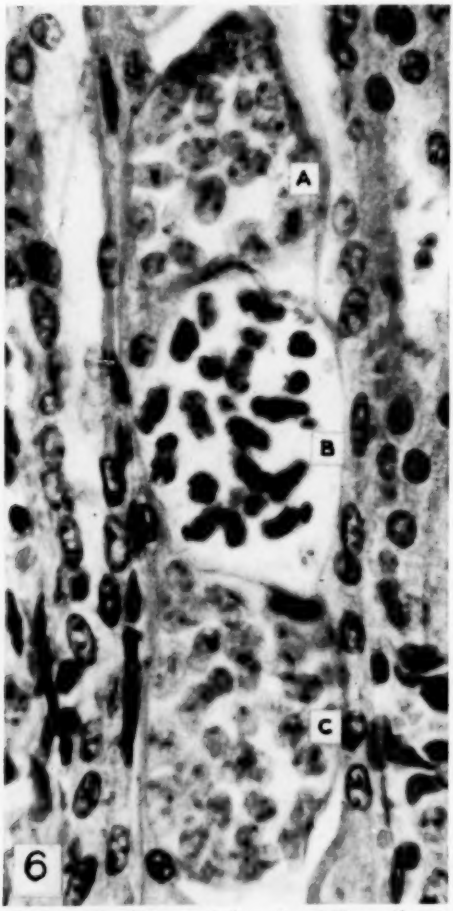

Fig. 6—Klossiella equi in zebra kidney tissue: oocysts with spores in different stages of development. Spores in (B) are more mature than are those in either (A) or (C). H & E stain; x 525.

An accurate count of spores could not be made; however, as many as 34 were counted in some of the larger sacs. Although different stages in spore development were observed, all the spores in any particular sac appeared to be in the same relative stage of development, indicating synchronicity. Numerous basophilic granules, seen in different locations within the spores, indicated nuclear division. A sporocyst wall subsequently developed, which had a strong affinity for basic dyes and obscured the developmental details of the sporozoites. However, as many as eight to 12 sporozoites were demonstrated in several sporocysts, which were especially stained for this purpose.

DISCUSSION

According to Kudo,[2] differentiation of *Klossiella muris* and *Klossiella cobayae*, found in the mouse and guinea pig, respectively, is made on the basis that the oocysts of the former contain 12 to 16 spores and the latter eight to 20 spores. Seibold and Thorson [4] counted as many as 40 spores in oocysts from the American jack and used this as a basis for establishing a new species, *Klossiella equi*. Baumann [1] had previously used this designation for a species in the horse, although he did not record any particular number of spores. However, his illustrations depict more than 20 spores each. In the zebra material, as many as 34 spores were counted in several oocysts, but the possibility exists that more than this number of spores might have been present in other oocysts which were not examined. Thus, there appears to be no clearly established basis for suggesting a new species in this instance. In the opinion of the authors, comparison of the parasitic forms in the kidney of the zebra with published descriptions and illustrations of *K. muris*, *K. cobayae*, and *K. equi* warrant the conclusion that the parasite in the zebra is *K. equi*.

Of particular interest was the occur-

rence of small eosinophilic masses within vacuoles of the uninuclear form. Baumann [1] apparently observed these in his material and interpreted them to be microgametocytes and the larger uninuclear form, the macrogametocyte. There is the additional possibility that these smaller masses might represent dissociated nuclei of the multinuclear form. However, the consistent size, location, morphology, and number (1 to 4) of these bodies militate against the latter possibility. From our observations, the impression was gained that these minute forms do represent separate bodies and might well be microgametocytes, as Baumann suggested. Various authors [1,3,6] suggest that some of the forms observed by them might represent schizogony stages. Seibold and Thorson [4] did not report any schizonts in the American jack, nor were any of the various forms observed by us interpreted to represent schizogony stages. Additional cases will be required to determine whether Klossiella spp. are one-host parasites or whether separate hosts are required for completion of the life cycle.

SUMMARY

Klossiella equi from the kidney of a zebra is described. Histological aspects of various stages in the sporogonic cycle are illustrated and briefly discussed.

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Postparturient Bovine Uterine Motility—Normal and After Experimentally Produced Retention of the Fetal Membranes

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RETENTION of the fetal membranes is still a considerable problem in bovine practice. Infectious agents often have been implicated as the cause of this condition, but many cases are not the result of infections.

The experiment reported here was designed to study the normal postparturient uterine motility pattern in the cow and to determine if an altered motility pattern accompanied experimentally produced retention of the fetal membranes.

REVIEW OF LITERATURE

Noninfectious retention of the fetal membranes was reported by Kennedy [9] in 8.3 per cent of cows from English herds where brucellosis was absent or quiescent. Palmer [12] and Boyd and Sellers [4] reported retention of fetal membranes in 11.7 per cent of 125 calvings and 6.4 per cent of 450 calvings in brucellosis-free herds. Fincher [5] observed that approximately one third of the cases of retained fetal membranes presented to the ambulatory clinic at Cornell University, Ithaca, N.Y., could be attributed to brucellosis; however, the cause of a large percentage of the cases was unknown, and many factors were implicated as contributing to this postpartum abnormality.

Jordan [8] reported a uterine motility study of 107 postparturient, brucellosis-free dairy cows, using an intrauterine balloon technique to record uterine contractions. Forty-three recordings of the normal uncomplicated puerperium demonstrated strong, rhythmic contractions at an average rate of 13.3 per hour during the first 12 hours, after which they averaged 7.9 per hour. Included in his report were 3 cows with retained fetal membranes in which uterine contractions were greatly reduced or entirely absent during the first puerperal hour. From these observations, Jordan hypothesized that retention of the fetal membranes was associated with a reduced uterine motility during the first 24 puerperal hours.

Benesch [2] reported uterine motility in the normal bovine puerperium, as recorded by an intrauterine balloon technique. Rates of 16 to 20 contractions per hour were observed initially, with a progressive reduction in rate and amplitude until contractions disappeared after the fourth day. Contractions were not observable on rectal examination after 48 to 60 hours. Benesch also observed that suckling by calves increased the uterine tone and accelerated and strengthened the contractions.

McDonald *et al.*[11] experimentally produced retention of the fetal membranes in cows by ablation of the corpora lutea. Of the 8 cows in the experimental group, 7 delivered calves before the end of normal gestation periods (278 days [7]) and 6 of these 7 delivered outside of one standard deviation from the mean gestation length.

MATERIALS AND METHODS

Uterine contractions were recorded, using a 4-inch rubber bag from which changes in pressure were transmitted through ⅜-inch tygon tubing to a rubber tambour. The intrauterine portion of the tygon tubing contained a 2.33-ft. length of ⅛-inch copper tubing, which helped to maintain the system along the normal curvature of the uterine horn and aided in holding it in place.

Recording periods were not begun until after two hours postpartum, since difficulty was encountered in maintaining the equipment in the uterus immediately after parturition due to uterine dilation, rapid uterine involution after expulsion of the fetus, and the cows' frequent and persistent straining.

The intrauterine segment of the recording equipment was sterilized chemically and placed manually in the recently pregnant horn. The air system of the recording device was exposed to the atmosphere for five minutes to allow the bag to adjust to the shape and size of the uterine lumen. During this period, equilibrium was reached between the negative abdominal pressures, the uterine tone, the elasticity of the sides of the rubber bag, and the atmospheric pressure. The system then was attached to the rubber tambour and contractions were recorded for varying periods of between 30 minutes and four hours.

Additional air sometimes was needed to obtain an interpretable recording. The potential large size of the bag aided in this adjustment without radically increasing the pressure inside the bag. These adjustments were necessary where the uterine lumen had a potential large size or where the uterus was inflated. The criterion used in determining the amount of air placed in the bag was the size of the excursions of normal respiratory movements on the kymograph.

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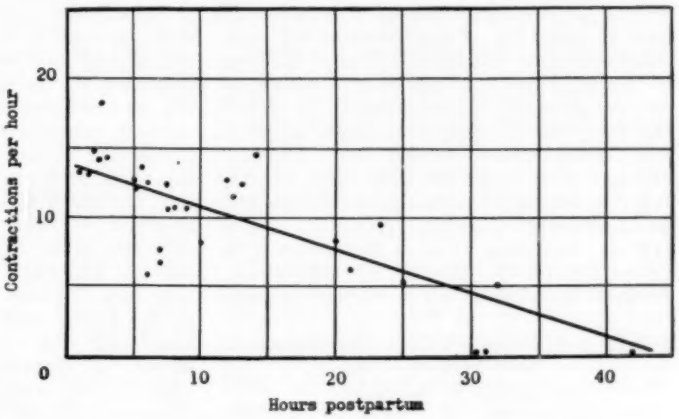
Based in part on a thesis submitted by the senior author to the Graduate School of the Oklahoma State University in partial fulfillment of the requirements for the M.S. degree.

The authors acknowledge the cooperation of the Department of Dairying during this study.



Graph 1—Rates of uterine contractions for normal cows.

It was found that if respiratory movements were recorded, uterine contractions, if present, were also recorded. If respiratory movements were not recorded, small uterine contractions were missed or were recorded at such low magnitude that there was doubt as to their validity when the tracings were studied.

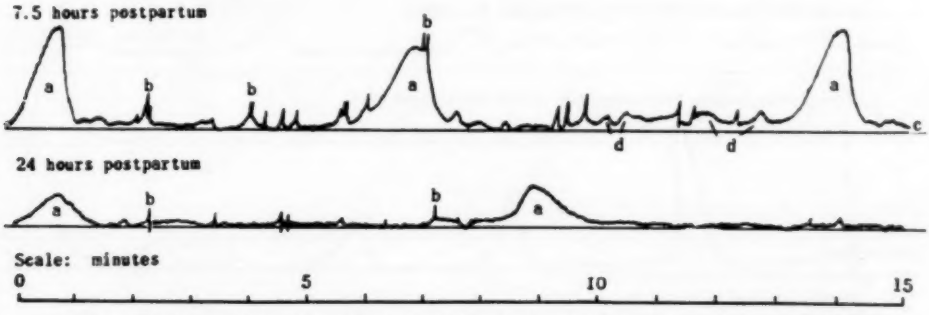
It is acknowledged that the amount of air in the bag affected the recorded force of the contractions. For this reason, studies were largely confined to the rate of contractions, since this apparently was not affected by the varying amounts of air used. Generally, between 25 and 50 cc. was used. The base line pressure varied from −5 to +10 mm. Hg.

The first study established the rate of uterine contraction during the normal puerperiums of 19 Holstein-Friesian, Jersey, and Guernsey cows of varying ages in the Oklahoma State University herd. Recordings were made during various periods of the puerperium, with data taken from a total of 30 recordings. Recordings were divided into 30-minute intervals, and each interval was studied in terms of rate, amplitude, and duration of contractions.

The second study consisted of postparturient tracings from 6 Holstein-Friesian cows whose corpora lutea had been removed between the 215th and 242nd day of gestation, a procedure designed to produce experimental retention of the fetal membranes without producing abortion. All of the cows were pluriparous, dry, and negative to a serum-agglutination test for brucellosis. The corpora lutea were removed by the intravaginal route described by McDonald.[10] After delivery, three daily recordings were made.

Results

Normal Bovine Uterine Motility.—The puerperal motility pattern of the group of normal dairy cows was characterized by rhythmic contractions occurring at an average initial rate of 14 per hour. This rate diminished as the puerperium progressed until, at 42 hours postpartum, the average rate of contraction was less than one per hour. During the first few hours of the puerperium, the contractions were



Graph 2—Uterine motility pattern for a normal cow, showing (a) primary uterine contractions, (b) skeletal muscle movements, (c) small respiratory movements, and (d) unrelated smooth muscle contractions.

strong, producing pressures of between 20 and 40 mm. Hg. Pain accompanied each contraction, as indicated by rapid shifting of the hind feet. As the rate subsided, so did the strength of the contractions. After the forty-eighth puerperal hour, motility was indiscernible, except for irregular changes in uterine tone. The rates of contraction during 19 normal puerperiums are summarized graphically (graph 1), and a typical recording from a normal cow is shown (graph 2). The average simple contraction lasted from 1.0 to 2.5 minutes and did not vary markedly throughout the first 32 hours of the puerperium. Long, weak contractions of three to four minutes' duration were found during the second day and may not have been physiologically identical to the distinct, strong contractions found during the first 24 puerperal hours.

TABLE 1—Histories of 6 Cows After Removal of Their Corpora Lutea

Cow	Corpus luteum removed (day of gestation)	Gestation length (days)	State of fetal membranes
1	242	274	Retained
2	221	266	Retained
3	215	219*	Retained
4	236	253	Retained
5	231	275	Retained
6	233	275	Expelled

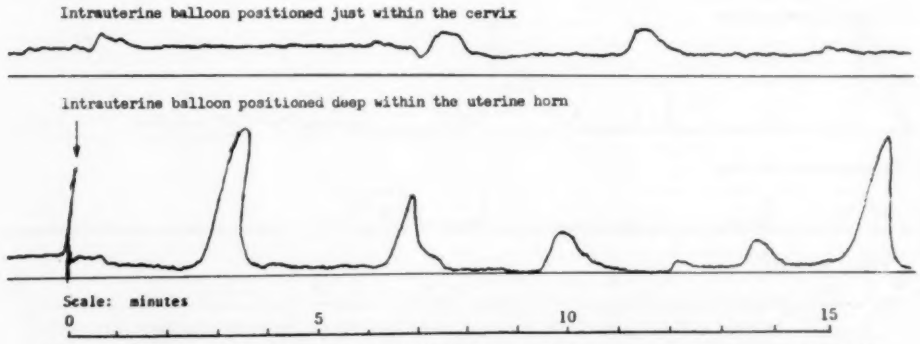
* Fetus aborted.

The rate and amplitude of the normal contractions were not affected by changes in the volume of air in the intrauterine bag. The base line was raised on several occasions to 20 mm. Hg without inducing a contraction or producing any noticeable difference in rate of contraction.

The effect of nursing on uterine motility was observed in 6 animals. In 4 of these, the rate of contraction and the amplitude remained the same or increased less than one contraction per hour. Two cows showed marked stimulation. One of these was nursed for 29 minutes. Two minutes after the onset of nursing, the rate of contraction increased from eight to 25 per hour and did not return to normal until 40 minutes after the end of the nursing period. The amplitude of the contractions was high (40 mm. Hg) before the nursing period began, and no further increase in amplitude was recorded. The other cow showed a stimulatory effect during an eight-minute nursing period. Four minutes after the calf started nursing, the rate of contraction increased from 11 to 30 per hour, and the amplitude was increased from 10 to 30 mm. Hg. The rate of contraction dropped to normal within 15 minutes after the end of the nursing period, but the increased amplitude persisted for 30 minutes.

Motility recordings were made from the recently pregnant horn, but occasionally the recording equipment slipped back into the body of the uterus where contractions could be recorded only after excessive inflation of the rubber bag. Recordings from two areas within the same uterus are compared (graph 3).

Uterine Motility of the Experimental Group.—Of the 6 experimental cows, 1 aborted four days after ablation of the corpus luteum. The 5 remaining cows had average gestation periods of 268.4 days, with a range of 253 to 275 days. Of these 5 cows, 4 retained the fetal membranes until liquefaction occurred or until they



Graph 3—A comparison between the motility patterns of the upper and lower segments of the bovine uterus.

were removed manually after the third puerperal day. One expelled the fetal membranes four hours after parturition. Data concerning the time of removal of the corpora lutea and the results thereof are presented (table 1).

The motility pattern during the puerperiums of cows in the experimental group differed markedly from that of the normal group. The rates of contraction in these animals were recorded on three successive postparturient days (graph 4). Abnormalities observed in the motility patterns were: (1) an increased rate of contraction, (2) a prolongation of the contractility of the uterus over a three-day period, (3) the appearance of tetanic contractions, and (4) the appearance of secondary superimposed undulations. A typical recording from a cow in the experimental group is shown (graph 5).

On the first puerperal day, the cow that expelled the fetal membranes showed a rate of contraction comparable to that of a normal cow whereas, on the second and third puerperal days, the rate did not decline as it did in the normal animals. The other experimental cows developed initial rates of contraction well above the normal and all maintained these rates through the three-day experimental period.

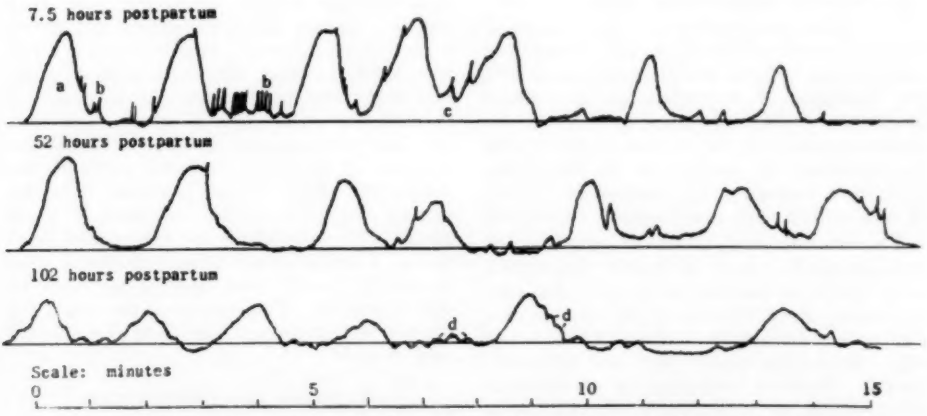
It was evident that those cows which had the earliest calving dates maintained the most persistent and accelerated motility patterns. In the cow that aborted, recordings were made through six puerperal days and the uterine contractions were as strong, rapid, and distinct on the sixth day as on the first.

Tetanic contractions were seen in all experimental cows, but there were individual variations. The animals that calved earliest had the greatest degree of tetany. The cow that expelled the fetal membranes had the longest gestation period and the least tetany.

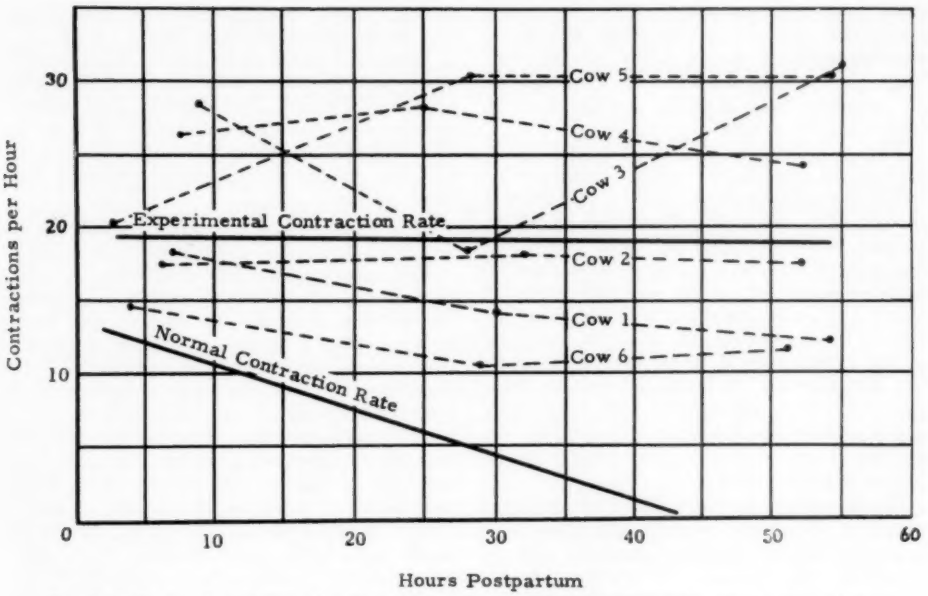
DISCUSSION

The uterine motility patterns of the cows in the normal group closely resembled the pattern described by Jordan and Benesch. Jordan's studies indicated a reduction in motility in direct proportion to time, but did not establish a limit within which puerperal uterine contractions could be recorded by the intrauterine balloon technique. Benesch's work demonstrated motility as late as the fourth day; those contractions recorded after the sixtieth hour were evident on his tracing, but were indistinguishable *per rectum*. The data reported here demonstrate a 48-hour limit to "easily identified" contractions. No recordings were made after the seventy-second puerperal hour in this study, but no contractions comparable to those reported by Benesch were found in the 48- to 72-hour period. Human beings,[3] in contrast to cattle, maintain recordable puerperal contractions for ten to 15 days.

Since the recorded contractions were maximum in rate and amplitude immediately after labor, it may be assumed that these puerperal contractions were continu-



Graph 4—Uterine motility patterns in cows with experimentally produced retention of the fetal membranes, showing (a) uterine contraction, (b) skeletal muscle movements, (c) development of tetany, and (d) secondary superimposed undulations.



Graph 5—Rates of uterine contractions in cows with experimentally produced retention of the fetal membranes.

ations of labor contractions. A different type of recording apparatus will be required to record the entire contraction pattern from parturition until the return of the normal pattern of estrus.[6] The appearance of secondary, superimposed undulations and irregular changes in uterine tone during the puerperium suggested reorganization of uterine motility in response to this changing physiological state.

The difference observed in the degree of uterine motility found in the horns and body of the uterus was in agreement with the findings of investigators in human medicine;[1,13] *i.e.*, increased motility in the upper segment of the uterus coupled with the reduction of motility in the lower segment was necessary for normal relaxation of the cervix and spontaneous childbirth.

The stimulatory effect of nursing on uterine motility in 2 of 6 cows indicates a complexity of factors governing this phenomenon. Jordan's study did not demonstrate an increase in motility when a calf was allowed to nurse the cow intermittently. Benesch reported a stimulatory effect, with no description of the variability of the response. The variability of the effect in this study may be a function of the vigor of the suckling as well as of the psychic state of the cow.

There was a delayed uterine involution in those animals which retained the fetal membranes, but the data (graph 5) show an increased uterine motility. This raises a question regarding the relationship between uterine motility and involution.

The motility pattern established by the experimental group and the cow that aborted during Jordan's study presented a picture of increased motility and a tendency toward tetany. The immediate hypothesis suggested to explain the increase of rate in the experimental group was that the low progesterone level caused by the removal of the corpora lutea reduced the inhibitory effect of that hormone, thus allowing the myometrium to respond more readily to circulating estrogens and oxytocin. Clinical observations indicate that progesterone inhibits uterine activity in late gestation. This has been the basis for its use in threatened abortion in women[13] and cows.[14]

SUMMARY AND CONCLUSIONS

The intrauterine balloon technique was used to study bovine uterine motility dur-

ing 19 normal bovine puerperiums and in 6 experimental cows with the corpora lutea removed, 5 of which retained the fetal membranes.

Normal puerperal uterine motility in the cow was found to consist, initially, of strong rhythmic contractions occurring at the rate of 14 per hour. These contractions diminished both in rate and in amplitude until they were unrecordable beyond the forty-eighth puerperal hour. Increased uterine motility was recorded during nursing in 2 of 6 normal cows.

Cows with experimentally produced retention of the fetal membranes had an increased motility pattern consisting of an average rate of 19 contractions per hour, which persisted throughout a three-day experimental period. Abnormalities in the contraction pattern of the experimental group consisted of tetanic contractions and the appearance of secondary superimposed undulations.

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Further Observations on Silage as a Possible Etiological Factor in Bovine Ketosis

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THE ASSOCIATION of grass silage with a high butyric acid content with bovine ketosis has been recognized by European workers.[11] The recognition of this relationship in New York State, and the identification of ketones in grass silage which contains excessive butyric acid,[1] led to a further study of the relationship of silage feeding and ketosis in dairy cows. Conrad and Hibbs [4] found the blood glucose levels of calves which were fed grass silage to be 45 to 50 mg./100 ml., whereas those of calves fed alfalfa meal and alfalfa hay were 55 to 60 mg./100 ml. When the ration of the calves which were fed grass silage was changed to mixed alfalfa and grass hay or Ladino clover pasture, there was an increase in the blood glucose levels to 55 to 60 mg./100 ml. The association of grass silage feeding with relative hypoglycemia further indicts it as a predisposing factor in ketosis.

A constant sign of ketosis in dairy cattle is a decrease in appetite as ketosis progresses; the silage is refused first, grain second, and hay third. A high concentration of butyric acid in strong, sour, or rancid-smelling grass silage is often involved as a factor in ketosis. Ketosis may occur at any time during the barn-feeding period, but is most prevalent in the late winter or early spring months when the contents of the lower portions of the silo are fed. In several herds, a number of cases of ketosis developed coincident with a change from corn silage to grass silage.

HISTORY

In late March of 1956, in a herd of 20 well-fed, high-producing cows that had seldom experienced ketosis in previous years, 4 cows developed the disease in rapid succession. They were treated with intravenous injections of dextrose, followed by oral administration of sodium propionate for five to seven days. Although the responses were favorable, relapses occurred promptly when the treatment was discontinued. These cows were again treated, in a similar manner, with comparable results. On the third visit to these patients, the therapy was changed to chloral hydrate *per os*, and the 2 most severely affected cows were also given 0.5 Gm. of hydrocortisone intramuscularly. After a few days of improvement, the cows relapsed again. The owner was greatly concerned because of the marked loss of condition and the severe inappetence of the affected cows. A review of the herd history revealed that, about one week prior to the onset of ketosis, the owner had fed the last of his corn silage and had started to feed a strong-smelling grass silage from the lower part of the silo. This was fed in large amounts, since the cows seemed to relish it. Previously, corn silage and good-quality hay had been used as roughage.

In view of the probable relationship between the silage feeding and the ketosis, the silage was withheld from the most severely affected cow and good-quality hay was substituted. All 4 cows were again treated with intravenous injections of dextrose and sodium propionate *per os*. The cow from which silage was withheld recovered promptly; the other 3 did not respond satisfactorily until their nutritional regimen was similarly changed. Their recovery was then satisfactory. Approximately two weeks later, when grass silage was again fed to these 4 cows, 1 of them experienced a relapse.

Ketosis was prevalent in another herd to which large quantities of grass silage and relatively small amounts of hay were fed. One cow failed to respond to treatment until good-quality hay was substituted for silage. Following this, the animal responded to sodium propionate therapy. About ten days later, after resumption of heavy silage feeding, the cow experienced a relapse.

During the past year, this relationship of grass silage to ketosis in dairy cattle has been observed frequently. A reduction in the quantity of grass silage and substitution of good-quality hay and grain in the ration is recommended for cows suffering from ketosis. This is also an important preventive measure for cows that are known to be predisposed to ketosis, especially where silage of high butyric acid content is being fed.

The pattern of favorable response to the reduction of grass silage feeding is not always consistent. Recovery follows its removal from the patient's diet in many instances. In other cases, the effect of feeding this silage is secondary to other factors, such as metritis. Under these con-

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TABLE 1—Mean Results of Assays of the Distillates

Identification	D_2* acetone (mg./kg.)	D_2 acetone (mg./kg.)	Acetaldehyde (μg./ml.)	D_3 acetone (mg./kg.)	Acetaldehyde (μg./kg.)	D_4 acetone (mg./kg.)	Acetaldehyde (μg./ml.)
Silo 1, grass	343.0	388.0	13.50	673.6	1.07	814.4	0
Silo 2, grass	359.5	338.5	26.30	364.0	0.87	376.0	0
Silo 3, corn	346.0	304.0	49.25	306.0	4.90	344.0	0
Silo 4, grass	44.0	29.6	31.56	29.6	3.55	29.6	Traces
Silo 5, corn	32.0	33.0	69.00	Traces	2.50	Traces	0
Silo 6, corn	80.0	62.0	103.00	27.6	6.30	Traces	Traces

* Initial determinations were performed on fresh samples; all other determinations were performed on poststorage samples.

ditions, the elimination of silage from the diet is not always followed by marked improvement.

In conjunction with these clinical observations, further studies on the ketone body content of silage were pursued.

MATERIALS AND METHODS

The method employed for the extraction and determination of the total ketone body content of silage has been described previously.[1] It has been reported that acetaldehyde in concentrations as low as 3 mg./100 ml. interferes with accurate determination of acetone when an alkaline salicylaldehyde solution is used as the indicator of its presence.[12] Since acetaldehyde and acetaldehyde precursors, such as ethanol and lactic acid,[2] are known to occur in silage, it was necessary to determine if and to what extent they interfere with the determination of ketone bodies by this method.

An all-Pyrex distillation system was used. It consisted of a 500-ml. flask with two outlets, one for connection with a condenser system and the other for connection with a reservoir for supplying water or other desired material to the contents of the flask. All connections consisted of standardized, ground-glass couplings.

Samples (20 Gm.) of silage were weighed as rapidly as possible and transferred to the distillation flask, which contained the desired volume of water. In 60 minutes, nearly 200 ml. of distillate was collected into a 200-ml. volumetric flask. Initially, this receiving flask contained sufficient water to submerge the lower end of the delivery tube leading from the condenser jacket, which prevented the escape and loss of acetone.

As a further precaution, the receiving volumetric flask was maintained in a cold-water bath during the distillation period. While the distillation was proceeding, water was allowed to drip into the distillation flask. Both the rate of water flow and the height of the gas flame were adjusted to maintain the water level in the distillation flask, and to fill the volumetric receiving flask almost to the volume mark during a distillation period of 60 minutes. At the end of this period, the contents of the collection flask were made up to volume by the addition of distilled water. This first distillate will be referred to as D_1.

An aliquot of D_1 was distilled, in preparation for the determination of total ketone bodies, according to the method of Behre and Benedict.[3] This second distillate will be referred to as D_2. In order to eliminate acetaldehyde from D_2, 25 ml. of Anstie's reagent[8] (an aqueous solution of potassium dichromate and sulfuric acid) was introduced slowly into the distillation flask. An aliquot of D_2 was introduced slowly into the flask from a pipette, the tip of which was submerged beneath the surface of the reagent. During the distillation, while the contents of the flask were boiling, 120 ml. of 0.2 per cent potassium dichromate solution was permitted to drip into the flask through the side inlet. The rate of flow was adjusted so that the total volume of this solution was delivered into the flask in 25 minutes. The distillation process was continued for another five minutes. Within this period of 30 minutes, a little less than 100 ml. of distillate was collected into the 100-ml. volumetric receiving flask. The distillate was then diluted to volume with distilled water. This distillate is referred to as D_3. Another distillate, to be known as D_4, was prepared from D_3 in a similar manner.

Distillates D_2, D_3, and D_4 were assayed for acetone and acetaldehyde contents. Ravin's[7] modification of the Behre and Benedict method was used for the determination of ketones. The acetaldehyde content was determined by treating the distillate similarly to blood-filtrate distillates, according to the method of Stotz.[10] Ketone bodies were computed on the basis of milligrams per kilogram, expressed as acetone. The acetaldehyde content is presented as *gamma* per milliliter of distillate. This latter determination does not give quantitative information relative to the acetaldehyde content of the silage. The sole object of this assay was to study its effect on acetone determinations.

Aliquots of D_2 from six silos were assayed for ketone body content immediately after D_1 was prepared. In order to determine the stability of these distillates, the remainder of D_1 was stored in the refrigerator in 125-ml. Erlenmeyer flasks. These were sealed with rubber stoppers which were covered with parafilm. In addition to this, parafilm was wrapped around the stoppers and necks of the flasks. The storage time ranged from eight to ten months. The samples were again distilled and assayed for ketone body content of D_2 at the expiration of this period, and the concentrations were found to be similar to the initial concentrations of D_2. Distillates D_3 and D_4 were also assayed for acetone content. All three distillates

were assayed for acetaldehyde. Each step, beginning with D_1, was carried out in duplicate.

RESULTS

The mean values obtained are presented (table 1).

The elimination of acetaldehyde by the oxidative action of Anstie's reagent was not achieved prior to D_4. In the D_4 distillation, it was clear that no interference occurred as a result of the presence of acetaldehyde. Similarly, diacetyl and formaldehyde can be excluded as interfering agents in D_4, since each interferes with the determination of acetaldehyde.[10] For the determination of acetaldehyde in D_2, it was found necessary to dilute the aliquot of the distillate 1:40, in order to obtain readings that did not exceed the significant range of the Coleman Junior Spectrophotometer employed. It was necessary to dilute D_3 1:2.

In those instances where the acetone content was high, *i.e.*, samples from silos 1, 2, and 3, there was no interference due to the presence of acetaldehyde in D_2. On the contrary, there was an increment in acetone content on subsequent distillations. This was particularly marked in the sample from silo 1. This increment was apparently due to oxidation of acetone precursors, such as *beta*-hydroxybutyric acid, isopropanol, and acetoacetic acid, which escaped oxidation to acetone during the initial distillation. Samples from silos 5 and 6, in which acetaldehyde interference was marked, were characterized by a low ketone body content, and the distillates were high in acetaldehyde content. The sample from silo 4 was low in both acetaldehyde and ketone bodies. In this instance, acetaldehyde interference was not apparent.

From the data obtained, it appears that, when the ketone body content of the silage is high, there is no significant interference with acetone determinations due to the presence of acetaldehyde. On the contrary, there is a marked tendency to interference of this nature with samples of low ketone body content. There are at least two causes of the phenomena. The first is physical in nature, and is due to the fact that there is proportionately more acetone in the solution; the second is biological, and is due to the fact that more lactic acid accumulates in samples which contain little butyric acid than in those which contain larger amounts of this substance.[2] It is the accumulation of lactic acid that causes a drop in pH and inhibits the butyric acid-producing organisms which are apparently responsible for the production of ketone bodies. Samples from silos 1 and 2 were characterized by a marked odor of butyric acid. In these instances, a 10- to 15-ml. aliquot of D_1 was sufficient to give significant readings in D_4 with a Coleman Junior Spectrophotometer, if 50-ml. aliquots were used for preparing D_3 and D_4. When the butyric acid odor is not present, 80-ml. aliquots should be used for all distillates. This was not the case with the sample from silo 3, in which no odor of butyric acid could be detected.

Acetone determinations on D_2 do not necessarily reveal the full amount of ketone bodies present when their concentration is high, and acetaldehyde interferes with acetone determinations when the silage contains low concentrations of ketone bodies. These limitations do not interfere

TABLE 2—Results of Assay of Silage Samples and Information on the Herds

Identification	Av. No. of lactating cows	Cases of ketosis	Ketosis in herd (%)	Ketones * (mg./kg. of silage)	Average annual milk production (lb.)	Breed of cows
Silo 1, grass	35	6	17.10	374.0	11,500	Holstein-Friesian
Silo 2, grass	25	3	12.00	410.0	11,000	Holstein-Friesian
Silo 3, corn	38	7	18.42	278.0	9,300	Holstein-Friesian
Silo 4, grass †	30	0	0.00	44.7	10,800	Holstein-Friesian
Silo 4, corn †	30	0	0.00	48.2	10,800	Holstein-Friesian
Silo 5, corn	20	0	0.00	33.5	10,000	Holstein-Friesian, 12 Jersey, 8
Silo 6, corn	76	8	10.50	83.5	12,000	Holstein-Friesian
Silo 7, corn	40	1	2.50	87.7	10,000	Holstein-Friesian
Silo 8, corn	40	0	0.00	61.0	9,000	Holstein-Friesian, 4 Guernsey, 36
Silo 9, grass	26	1	3.46	83.0	9,000	Guernsey, 36

* Expressed as acetone; † this barn had two types of silage.

with the distinction between high and low concentrations of ketone bodies in D_2. Determinations on D_2 appear to be satisfactory for clinical purposes.

The ketone body content of ten silage samples was determined by the methods described. All of the determinations were performed on D_2. Samples from each silo were assayed in duplicate, except the sample from silo 2, which was carried out in quadruplicate, and that from silo 6, in which material from only one determination was available. The mean values are presented (table 2). Table 2 also contains information relative to the type of silage, frequency of clinical ketosis in the herds from the various barns included in this study, the breed of cows, and the average annual milk production. The average milk production is based on records, with the exception of herds 5 and 7, from which the only available information was based on an estimate. All of the data presented are given for the year that included the season when the ketone determinations were performed on the silage samples.

Discussion

Various clostridia species are responsible for the fermentation of butyric acid and related compounds.[9] These organisms are ubiquitous and may flourish in silage.[2] Under certain circumstances, they cause the production of butyric acid in silage.[2] In properly prepared silage, lactic acid accumulates and, by the end of three weeks, the pH drops below 4.2. At this pH, the clostridia are inhibited. When the pH remains above 4.2, the clostridia become active and cause production of butyric acid in the silage.

The presence of these organisms in the silage presents the question of whether they can continue their activities in the rumen. The silo and rumen are both presented with similar substrates, both are anaerobic, and both are undoubtedly inoculated with clostridia. According to Masson,[5] *Clostridium butyricum* and Lactobacilli sp. predominate in the rumens of sheep when their diets contain flaked maize. The association of ketone bodies with butyric acid fermentation in silage is to be expected. The ketogenic characteristics of the butyric acid organisms are well known and have been applied to the industrial production of acetone.[6] For these reasons, the presence of significant quantities of ketones in silos 1 and 2 was not surprising, since a strong odor of butyric acid emanated from each of them. The presence of a high concentration of ketone bodies in the sample from silo 3 can not be explained on this basis; the silo contained corn silage and no odor of butyric acid was apparent. It seems obvious that some other type of ketogenic fermentation was taking place.

The authors have not found information in the literature relative to butyric acid fermentation in corn silage. It seems reasonable to expect that ketogenesis in grass silage should serve as an index of butyric acid production in this nutrient. Furthermore, ketogenesis in silage can serve as a source of extrahepatic ketones and explains, in part, the positive reactions to the Ross test that occur in healthy cows with normal blood glucose levels.

The three instances marked by a high ketone content of the silage were associated with a high incidence of ketosis in the herd to which this material was fed. The percentage of clinical cases of ketosis in these herds ranged from 12.0 to 18.4. The only herd studied which had a ketosis incidence approaching these values was herd 6, in which 10 per cent of the animals exhibited ketosis at some time. The annual average milk production of this herd was 12,000 lb. and, therefore, it should be considered in a special category, since high milk production is a well-known predisposing factor to ketosis. Other factors to be taken into consideration in a study of this nature are the proportion of silage to other roughage in the diet, and the quantity and type of grain supplement. Accurate data of this nature were not available in this study.

Summary and Conclusions

1) The presence of ketone bodies has been demonstrated in grass and corn silage.

2) The ketone body content of silage from different silos may vary significantly, with particularly high levels occurring in grass silage which contains excessive butyric acid. A high level of ketone bodies was apparent in corn silage in one instance.

3) There appears to be a relationship between the ketone body content of the silage which was fed and the frequency of ketosis in the herd. This is probably due to the fact that ketogenesis in grass

silage serves as an index of butyric acid production.

4) Clinical observations indicate that silage which contains butyric acid is a significant predisposing factor to ketosis.

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Nocardia Asteroides—A Newly Recognized Pathogen of the Mastitis Complex

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THE RECORDED HISTORY of nocardial infection stems from Nocard's original isolation [29] of an aerobic acid-fast actinomycete from cattle afflicted with "farcin de boeuf" in 1888. This organism is now designated *Nocardia farcinica*.[8] In 1891, Eppinger [16] reported a similar organism isolated from a human brain abscess which proved fatal to the host. This species is now designated *Nocardia asteroides*.

Nocardia asteroides infections have been the subject of numerous reports since Eppinger's original description of the species. Henrici and Gardner [22] reviewed 25 recorded cases of *N. asteroides* infection in human beings in 1921. Of the 25 infections, 16 were pulmonary; 5, cerebral; 2, postoperative peritonitis; 1, spinal abscess; and 1, pedal cellulitis. In a 1952 review, Cruz and Clancy [12] summarized 40 reported cases of "nocardiosis" in human beings, of which 33 involved the lungs alone or in combination with other organs. Of these 40 cases, 3 occurred in central and southern California. Only 9 of the 40 recovered; 6 of these since 1940, when sulfonamides were used in treatment.

The recent literature contains a series of articles, of interest to veterinarians, concerning canine nocardial infections.[5, 6, 11, 20, 32, 35] In a review of the disease in dogs and cats by Thordal-Christensen and Clifford,[32] the most frequently reported sites of localized infection are the cutaneous, subcutaneous, thoracic, and abdominal areas, with infrequent oral, nervous tissue, and osseous mandibular involvement. Trevino [35] describes nocardiosis as, " . . . the disease caused by *N. asteroides* . . . characterized by abscesses and granulomatous lesions of the lungs, skin, and lymph nodes." However, other species of Nocardia have been described as being capable of causing similar disease processes in both animals [20] and man.[13, 22]

Nocardia asteroides apparently was first isolated from cattle by Bishop and Fenstermacher in 1933.[4] They recovered the organism from necropsy specimens of diaphragmatic and pleural lesions in a cow suspected of having tuberculous infection.

Various species of Nocardia or Nocardia-like organisms have been studied by Traum [34] and others [19, 21] in relation to bovine lymphangitis and skin lesions in reactors to the tuberculin test. However, they did not specifically identify *N. asteroides*.

Evans,[17] in 1918, reported on a number of milk samples which contained an organism identified presumptively as *Streptothrix hominis* III (Claypole). (*N. asteroides* has been referred to by this name.[8]) However, the organism described differed in colony morphology and biological reaction from those currently accepted for *N. asteroides*. There was no clinical evidence of infection in the udders from which the infected milk came, with the exception of one quarter which shed large numbers of streptococci as well as the Streptothrix (Nocardia) reported.

In 1935, Gray [20] reported a case of bovine mastitis in England, which was caused by an organism similar to *N. farcinica*. The infection involved a single quarter of a mature cow. The quarter was enlarged, fibrotic, and febrile.

Two cases of bovine mastitis caused by *N. asteroides* are recorded; 1 was from Australia,[27] the other from Canada,[2] and both were single quarter infections. The Australian case cleared spontaneously after four and one half months. An animal experimentally infected with the organism isolated from this field case developed nodular fibrosis and thick-walled abscesses in the inoculated gland. The Canadian case produced no clinically detectable lesions.

This paper will discuss a series of mammary infections by *N. asteroides* encountered in a herd of dairy cattle in the southern coastal area of California. To the authors' knowledge, the disease has not previously been described in this country nor has it previously been shown to involve an appreciable segment of a single herd. A preliminary report on these findings has been published.[30]

DESCRIPTION OF THE ORGANISM

Aseptically drawn milk samples were streaked both fresh and after 20 hours of incubation at 37 C. on tryptose agar containing 5 per cent washed bovine erythrocytes. The colonies, which appeared after 20 hours of aerobic incubation at 37 C., were minute, whitish, slightly raised, and nonhemolytic. After 48 hours, the colonies were 1 mm. in diameter, raised, whitish,

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The cooperation of the owner of the infected herd and of Dr. D. H. Wixom, practitioner, are gratefully acknowledged. The authors also thank Dr. W. H. Rockey, California Bureau of Livestock Disease Control, for running the skin-sensitivity tests.

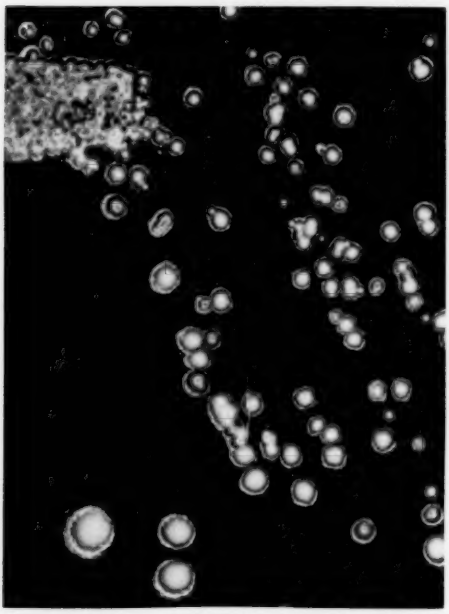

Fig. 1—Colonies of Nocardia asteroides on tryptose agar after 96 hours' incubation. x 10.

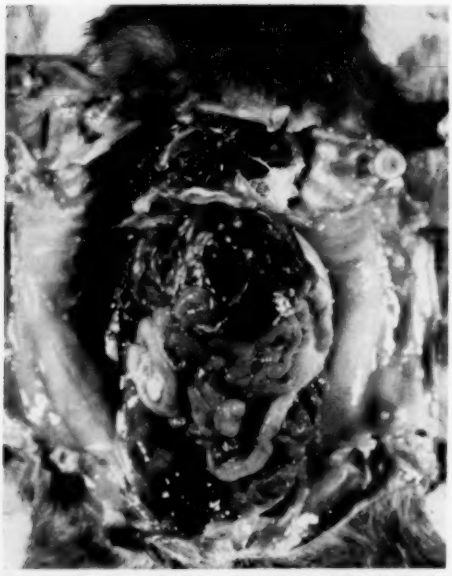

Fig. 2—Lesions of the peritoneum, abdominal viscera, and diaphragm of a guinea pig after intraperitoneal inoculation of Nocardia asteroides. Death occurred 96 hours after injection.

and adherent; they resembled colonies of *Micrococcus pyogenes*. Additional incubation produced opaque, convex colonies with slightly granular surfaces and translucent, mycelioid edges (fig. 1). Pigmentation of the colonies usually appeared by 72 hours; it varied from an initial light buff to a bright salmon color by the fourth week of incubation. Aerial mycelia were not observed.

The organism was gram-positive; fine mycelial elements, with and without branching, and shorter rod and coccoid forms were evident. In this laboratory, the organism from cultures has been essentially nonacid-fast. When the exudates from infected glands and guinea pig lesions were rapidly destained with 2 per cent H_2SO_4 (alcohol-free), the elements retained more of the carbol fuchsin than when acid alcohol, employed in the Ziehl-Neelsen method, was used.

The cultures were nonhemolytic to equine and bovine erythrocytes. No growth occurred on Edward's medium, MacConkey's agar, or mannitol-salt agar.

Growth reactions on differential mediums were as follows:

Methylene blue milk—early reduction (24 hours) with later return of the dye color which then remained.

Litmus milk—slow alkalinization, no coagulation or reduction in 28 days.

Dorset egg—no liquefaction occurred in 28 days; abundant waxy, nodular, salmon-colored growth.

Löffler's serum—no liquefaction or peptonization; buff-colored granular growth.

Dextrose agar shake—surface growth only.

Gelatin—no liquefaction in 28 days.

Serum broth—delicate waxy pellicle adherent to the sides of the tube and small, round, fluffy, mycelial masses at the bottom; intermediate zones were clear.

Potato agar—luxuriant growth; after 28 days, the colonies were more than 1 cm. in diameter, conical, waxy, nodular, and salmon colored.

Nitrate reduction was positive; indole was not formed. Methyl red and Voges-Proskauer reactions were both negative; catalase was formed. Hydrogen sulfide and ammonia were not produced; starch was not hydrolyzed. Neither acid nor gas was formed in serum-enriched lactose, sucrose, salicin, mannitol, glucose, or maltose cultures. The organism could utilize paraffin as a carbon source; this was of value when isolating the organism from soil samples by the method of Frey and Hagan.[19]

Animal Inoculation

Broth cultures (24- and 72-hour) were inoculated intraperitoneally into guinea pigs and mice. When guinea pigs were given 0.4- to 1.8-cc. inoculations, they all died in three to eight days, a much shorter time than is usually reported for this infection. The time of death was independent of the age of the culture, indicating little or no effect from preformed toxin. One pregnant female aborted three days after inoculation and died five days later.

Necropsy of the guinea pigs showed abundant 1- to 2-mm. raised, white lesions scattered over the parietal and visceral peritoneum (fig. 2). These foci were hard and caseous. Larger lesions were encountered at the inoculation site. Lesions were most abundant in the anterodorsal aspect of the peritoneal cavity between the liver, the peritoneal wall, and the diaphragm. The omentum and pancreas were usually heavily involved. Diaphragmatic lesions eroded through that organ, and infection of the lungs ensued; adhesions between serous surfaces and underlying lesions were frequent. There were many focal hepatic and pancreatic lesions, but little splenic and renal involvement. The organism was cultured from numerous lesions, peritoneal and thoracic fluid, lung and liver parenchyma, splenic pulp, and the heart blood of various guinea pigs.

A wet mount of the exudate from one of the lesions is shown (fig. 3). The pus was somewhat granular, but the granules appeared to be mycelial masses rather than the true "sulfur granule" or rosette of actinomycosis.

Mice were not visibly affected by inoculation. When they were killed, 24 days postinoculation, only occasional lesions were found. The organism was recovered from an omental nodule, a kidney infarct, and a lesion in the wall of one uterus.

From this description of the biological and pathogenic reactions of the organism, it is apparent that this infectious agent is either *N. asteroides*, as described,[8] or a closely related species (or strain), *Nocardia variabilis*,[25] which has been found to be nonacid-fast.

Cultures were submitted to two laboratories for identification. The organism reacted in high titer with *N. asteroides* antiserum at one laboratory[14] and was identified as *N. asteroides* by the other.[1]

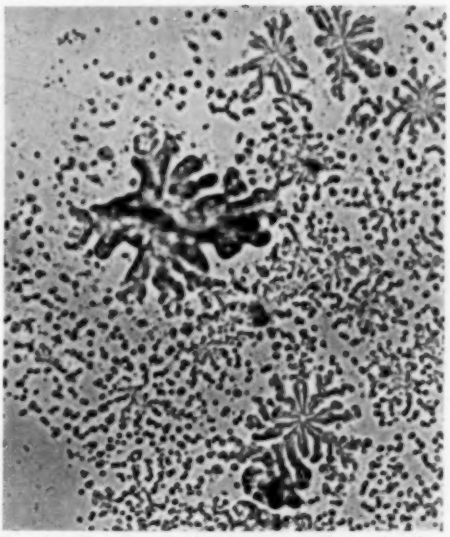

Fig. 3—Wet mount of infective exudate, showing mycelial masses. x 320.

This second laboratory found the mycelia isolated from infected guinea pigs to be acid-fast.

Susceptibility of the Organism to Heat, Antibiotics, Chemotherapeutics and Disinfectants

Serum broth cultures (24-hour) were placed in water baths of varying temperatures and subcultured after 15 and 30 minutes' exposure. Subcultures were positive after 30 minutes' exposure to 64 C., but no growth was obtained after 30 minutes at 66 or 68 C. Sterile milk cultures treated similarly also showed that the organism survived 30 minutes at 64 C., but not 30 minutes at 66 C.

The organism's response to several commonly used disinfectant solutions was tested (table 1). The diluted solutions were inoculated with a 72-hour broth culture, thoroughly mixed, and subcultured on tryptose agar plates after five- to 90-minute exposures at room temperature.

Drug-sensitivity testing, using commercially available paper test discs and compressed tablets, revealed that the organism was susceptible *in vitro* to 30 μg. of novobiocin (Albamycin*). Nitrofurazone (Fur-

* Albamycin (novobiocin sodium) is produced by the Upjohn Co., Kalamazoo, Mich.

TABLE 1—Response of Nocardia Asteroides to Several Commonly Used Dairy Disinfectants

Disinfectant	Exposure time (min.) 5	10	30	60	90
Chlorine (p.p.m. available Cl)					
100	—*	—	—	—	—
200	—	—	—	—	—
400	—	—	—	—	—
Benzalkonium chloride (Roccal †)					
100 p.p.m.	±*	—	—	—	—
200 p.p.m.	—	—	—	—	—
400 p.p.m.	—	—	—	—	—
Ethanol and glycol iodine complexes (Iosan**)					
50 p.p.m.	+*	+	+	+	+
100 p.p.m.	+	+	+	+	+
200 p.p.m.	+	+	±	+	—
Chlorhexidine diacetate (Nolvasan ‡)					
100 p.p.m.	+	+	+	+	+
200 p.p.m.	+	+	+	+	+
400 p.p.m.	+	+	+	±	±
4,000 p.p.m.	±	±	±	—	±

* — = no growth, ± = growth reduced, and + = normal growth; † Roccal (benzalkonium chloride) is produced by Winthrop Laboratories, New York, N.Y.; ** Iosan (ethanol and glycol iodine complexes) is produced by Lazarus Laboratories, Long Island City, N.Y.; ‡Nolvasan (chlorhexidine diacetate) is produced by Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.

acin,† 100 μg.) and erythromycin (10 μg.) also exhibited some effectiveness, while three tetracycline derivatives (5 μg.), chloramphenicol (5 μg.), penicillin (1 unit and 10 units), polymyxin (5 units), neomycin (5 μg.), and streptomycin (10 μg.) were without effect.

THE DISEASE IN CATTLE

Culturing of milk samples from the affected herd has shown a mixture of infection. In addition to the Nocardia, isolations of *Micrococcus pyogenes, Streptococcus agalactiae,* and *Pseudomonas aeruginosa* have been made.

Nocardia asteroides has been isolated in pure culture or in combination with one or more of these organisms on from one to three occasions, from 28 of 157 animals examined in this herd. Clinical evidence of infection frequently was encountered shortly after the animal freshened, and the organism has been isolated from several animals within seven to ten days after calving. Positive isolations have been made from the udders of 2 first-calf heifers, 1 of which showed clinical involvement during her first week of lactation.

† Furacin (nitrofurazone) is produced by Eaton Laboratories, Norwich, N.Y.

Affected animals may have anorexia and fevers up to 107 F.; however, only a single fatality has been observed. Clinical evidence of infection is frequently restricted to mammary inflammation, denoted by strongly positive reactions to the California mastitis test (CMT),[31] shedding of clots of exudate, and fibrosis of the infected gland. Palpation of the glands often reveals a diffuse firmness or, more typically, the development of discrete nodules 2 to 5 cm. in diameter.

Exudate from the infected glands was grayish and viscid. Small white particles about 1 mm. in diameter were scattered throughout; microscopically, they were composed of dense masses of mycelia.

A severely affected first-calf heifer, which had shown clinical evidence of infection since her first week of lactation, was killed for necropsy. The organism had been recovered from her udder in pure culture on three consecutive occasions; three of the mammary quarters were involved.

Grossly, the udder showed areas of diffuse fibrosis in the dorsoposterior regions of the right rear quarter. Several thick-walled abscesses, 2 to 5 cm. in diameter, were prominent in the lower portions of the gland, and lesser lesions were scattered throughout the secretory tissue. The lining of the teat sinus was thickened and rough, causing a granular or pebbly appearance (fig. 4). The lining of higher ducts was similarly involved in various areas.

The right supramammary lymph node, which drained two infected quarters, was twice normal size. The cut surface was moist, but neither gross nor microscopic lesions were encountered. No involvement was detected in the internal inguinal, sublumbar, mesenteric, or mediastinal lymph nodes, or in the spleen.

Nocardia asteroides was cultured from the milk of the right fore and rear quarters, from the left rear quarter, and from the tissues of these glands. Macerated tissues from these lymph nodes and the spleen were uniformly negative on culturing.

Tissue sections from the infected glands were studied microscopically. One of the larger lesions from the right rear quarter is shown (fig. 5, 6). The granulomatous nature of these foci is quite evident. The center of the lesion was comprised of necrotic debris, polymorphonuclear and mononuclear phagocytes, lymphocytes, and plasma cells; giant cells were not seen. A

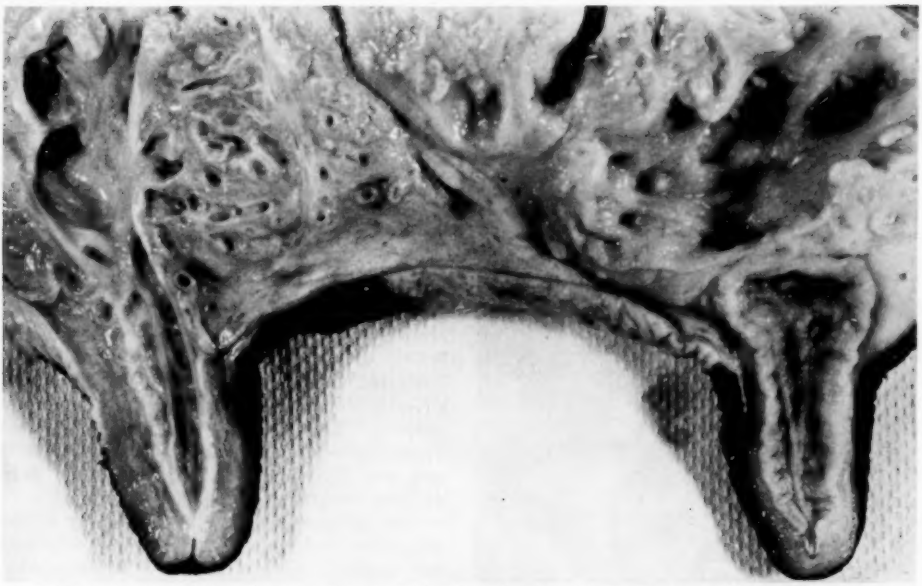

Fig. 4—Bovine udder, showing gross pathological changes after experimental Nocardia asteroides infection. Notice the thickened granular appearance of the lining of the infected gland at the right; normal gland (left forequarter) to the left.

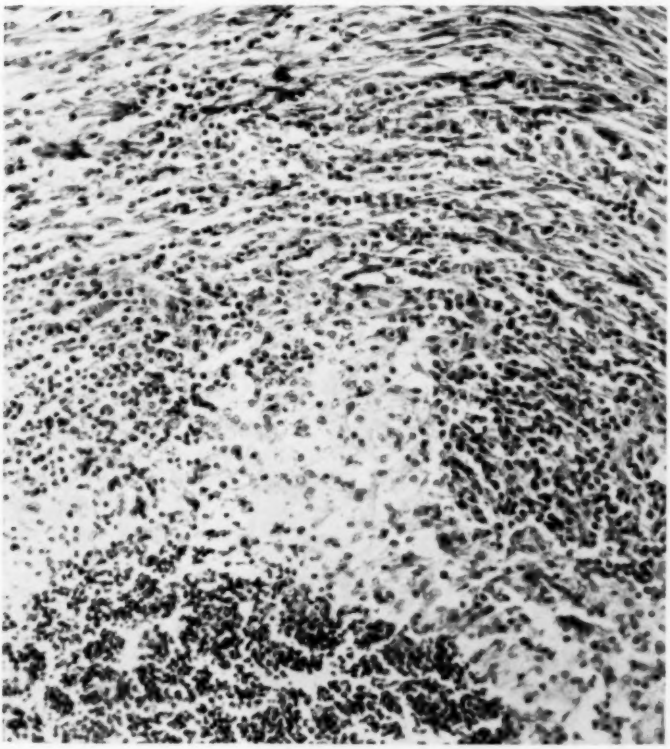

Fig. 5—Section of a naturally infected udder, showing histological change: a thick-walled granuloma with necrotic center, typical of the larger palpable lesions. H & E stain; x 100.

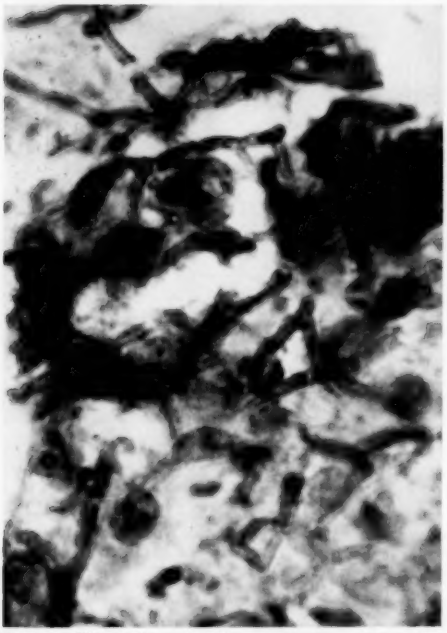

Fig. 6—Nocardia asteroides organisms, from the center of the lesion shown in figure 5. This section was stained for acid-fastness, but the mycelia appeared nonacid-fast. Ziehl-Neelsen stain; x 900.

band of epithelioid cells surrounded the central necrotic area and, around this, there was an extensive fibrous capsule infiltrated by a variety of leukocytes. Such lesions were palpable in the intact udder.

In a smaller lesion (fig. 7), the band of epithelioid cells around the necrotic center was prominent, while the fibrous capsule had not yet developed.

Hypertrophic and hyperplastic changes occurring in the glandular epithelium are shown (fig. 8). These cells were vacuolated, greatly elongated, and had nearly occluded the lumen. Plasmacytic and lymphocytic infiltrations into this area are evident. This reaction and the roughened appearance of the lining of the teat sinus suggest the presence of irritating substances resulting from the infection.

The appearance of the glandular tissue suggested a lobular involvement; one lobule showed little or early involvement, while an adjacent lobule was nearly unrecognizable due to the completeness of destruction of its secretory elements (fig. 9).

EXPERIMENTAL INFECTION

A strain of *N. asteroides* isolated from the right rear quarter of this animal was inoculated into two quarters of an experimental cow during her first lactation. One cubic centimeter of 24- and 72-hour serum broth cultures was inoculated into the left

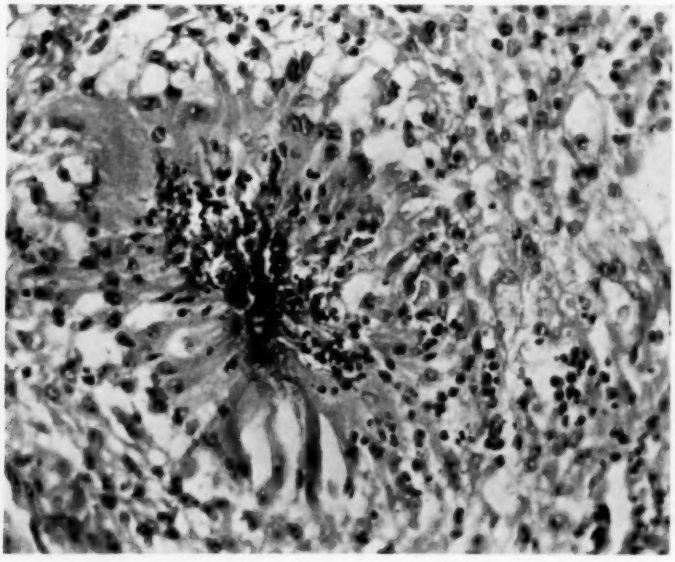

Fig. 7—Microscopic lesion from a naturally infected udder. The epithelioid band is prominent about this early focus, while the fibrosis about the periphery is not as advanced as that shown in figure 5. H & E stain; x 400.

fore and right rear quarters, respectively. Control infusions of sterile serum broth were made into the remaining two glands.

Twenty-four hours later, the inoculated quarters were both shedding visibly altered milk. The milk from the control quarter on the right side appeared normal, but reacted strongly to the CMT.[31] The milk from the control quarter on the left side was visibly normal and negative to the CMT. Rectal temperature remained normal.

By the second day postinoculation, the cow's temperature was 104.8 F. Milk from both inoculated quarters contained strands of pus, while the two control quarters gave milk which appeared normal, but reacted positively with the CMT. Total milk yield was considerably reduced.

By the fifth day, the infected quarters were enlarged and hard, the animal was depressed, off feed, and had a temperature of 106.6 F. Fearing a fatal termination, 500 mg. of novobiocin in a 20-cc. sterile diluent was infused into the left fore quarter, and 200 cc. of 25 per cent sulfamerazine solution was administered by venoclysis at 24-hour intervals for three days. The rectal temperature then dropped steadily, and was 101.8 F. five days later. The treated quarter, which had shed *N. asteroides* continuously since inoculation, became negative on culturing for two consecutive days following beginning of therapy, but was unimproved clinically. The untreated quarter continued to shed the organism during this period. The organism subsequently was reisolated from the treated gland on numerous occasions, and the febrile reaction returned and remained until the cow was killed 33 days postinoculation. During this period, the animal lost 370 lb. in body weight.

Periodic blood counts during the course of infection revealed a progressive increase in the percentage of neutrophilic leukocytes. Band cells reached 8 per cent of the

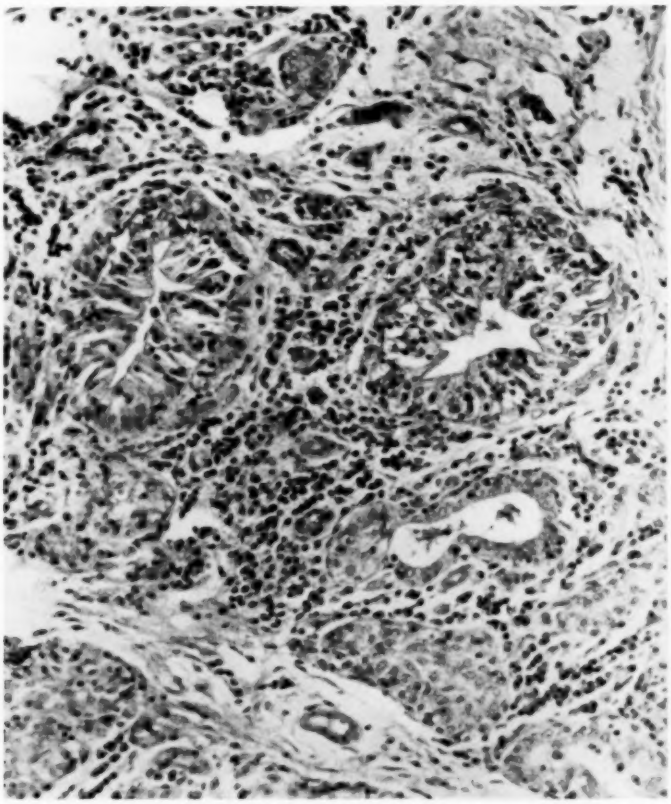

Fig. 8—Section from a naturally infected udder, showing hypertrophic changes in the glandular epithelium, which is nearly occluding the lumen. H & E stain; x 100.

differential count and segmented neutrophils 60 per cent by the seventh day of infection. On postinoculation day 5, the eosinophil count was 13 per cent of the differentiated cells; subsequent examinations showed that eosinophils remained at the 1 per cent level. The total white cell count did not exceed 10,250 cells per cubic millimeter.

Necropsy findings were similar to those seen in the field case, with the exception that the pathological changes in the experimentally infected glands were more extensive than in natural infection. The linings of the teat sinuses of the infected quarters again were thickened and granular. The inoculated glands were enlarged and hard. Two adjacent foci appeared to have penetrated the intermammary septa and invaded the noninoculated gland (fig. 10). At no time did culturing of the milk from either control gland reveal the presence of Nocardia. The extreme fibrosis of the infected gland is grossly visible.

The supramammary nodes were enlarged by 50 per cent and the internal inguinal nodes were also enlarged and edematous. Gross and microscopic lesions were seen, and the organism was recovered only from the mammary tissue proper.

THERAPY TRIALS IN NATURALLY INFECTED ANIMALS

In the literature reviewed, 6 of the 9 reported recoveries from nocardial infections in human beings seemingly were the result of sulfonamide therapy. Harrell [23] states, "It is unfortunate that some investigators are recommending penicillin and other antibiotics, such as streptomycin, in the treatment of nocardiosis." He recommends [24] sulfadiazine as the most effective drug but only after full therapeutic doses are maintained for many weeks.

Our *in vitro* sensitivity tests indicated that two of the drugs tested (nitrofurazone at 100 μg. and novobiocin at 30 μg.) might show clinical efficacy. In addition to these, mercumatillin sodium (Cumer-

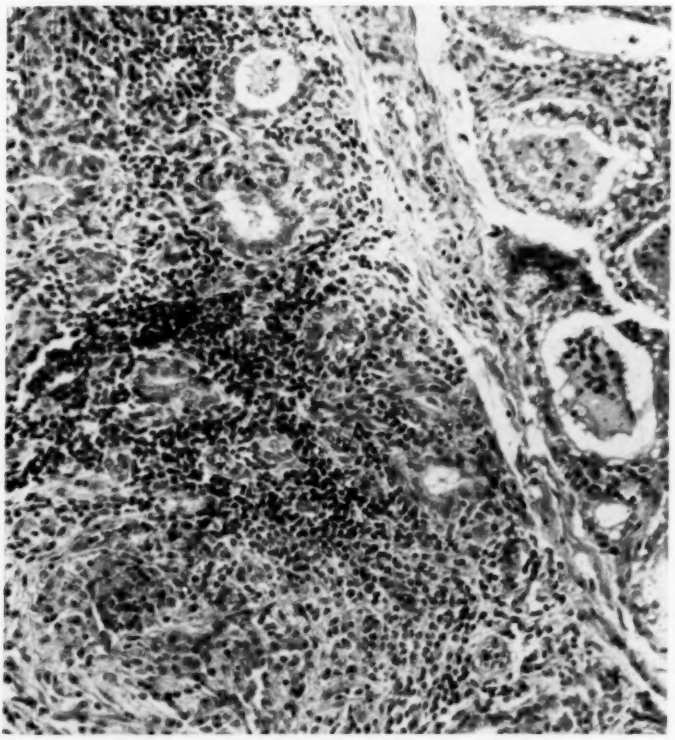

Fig. 9—Pathological changes in a naturally infected mammary gland. This section shows typical lobular distribution. H & E stain; x 100.

tilin sodium *) at 100 μg. per milliliter and bis-(4-aminophenyl) sulfone† at 10 μg. per milliliter showed good *in vitro* inhibition of the test strain of *N. asteroides*.

Nitrofurazone solution (0.2%) was already in use as a therapeutic agent in this herd. Treated cows did not improve clinically and continued to shed the organism but, as long as the treatment was continued, the process did not progress but stayed "fixed" at that level. Some animals may have stopped shedding Nocardia as a result of nitrofurazone therapy, but the data are insufficient and the drug was used too promiscuously to definitely establish this point.

Novobiocin (500 mg.) was combined with 25 to 40 cc. of nitrofurazone solution (0.2%) and infused into selected quarters twice daily.

A résumé of 4 treated animals follows:

Cow 126, C Quarter.—Nocardia were isolated on April 1 and April 16. Treatment, beginning April 17, consisted of 500 mg. of novobiocin in 25 cc. of nitrofurazone solution, administered twice daily by intramammary infusion. Three such doses were given. The milk cultures were negative for Nocardia on April 25, May 7, and May 22.

Cow 165, All Quarters.—Nocardia were isolated from composite samples of all quarters on April 1 and April 16, and from individual quarter samples on April 11. Treatment was identical with that for cow 126. Milk cultures were negative for Nocardia on April 25. The cow was then marketed because of the extensive fibrosis of the udder.

Cow 105, C Quarter.—Nocardia were isolated on April 23. Treatment consisted of 500 mg. of novobiocin in 40 cc. of nitrofurazone solution, infused twice daily for five consecutive days, beginning April 23. Milk cultures were negative for Nocardia on May 7, May 22, and June 27.

Cow 137, A Quarter.—Nocardia were isolated from the milk on April 16, but were not found just prior to treatment on April 23. Treatment was the same as described for cow 105, but for four consecutive days only. Milk cultures were negative for Nocardia on May 7 and May 22.

The last 2 cows showed marked clinical improvement by the time of the last treatment. While the organism had been isolated from cow 105 only once prior to treatment, she had grossly palpable lesions in the gland, which were typical of the infection.

The ultimate duration of these infections is unknown; the possibility of spontaneous clearance of Nocardia from previously infected glands has been indicated.[27] It is however, the authors' opinion that the clinical and bacteriological response in these naturally infected glands, following the treatment described, merits its consideration in *N. asteroides* infection.

The experimentally infected animal discussed previously did not recover after a three-day treatment of novobiocin infusions plus intravenous sulfonamide, nor were four subsequent daily infusions of help. It is doubtful that any treatment short of surgical removal of the infected glands[20] could have benefited this animal (fig. 10).

SOURCE OF THE INFECTION

The ecological niche ordinarily ascribed to the genus Nocardia, when not residing in animal tissue, is the soil. Like other potential pathogens of similar distribution, (e.g., *Ps. aeruginosa*), its occasional presence in the bovine mammary gland might be expected. Yet this gland, which jealously nurtures a wide variety of parasites, has in only two other reported cases been invaded by *N. asteroides*. In the herd reported here, only two quarters shed *Ps. aeruginosa*, while 28 cows shed Nocardia from one or more quarters. Therefore, efforts were made to determine the vector by which the organism entered the mammary gland with such unusual frequency.

In the segment of the herd which was being treated by mammary infusion by the owner, it was found that, after cleansing the teats with alcohol, a single cannula was used repeatedly with only momentary immersion in alcohol between quarters. The alcohol was changed every 24 to 48 hours and, although it appeared rather turbid, there was no bacterial growth when it was cultured. There was, however, ample time for the alcohol to kill any susceptible organisms between the time the sample was obtained and the time it was cultured. This method of spreading the infection, while not established by cultural data, remains a possibility. It fails, how-

* Cumertilin sodium (mercumatillin sodium N.N.R.) is produced by Endo Products, Inc., Richmond Hill, N.Y.

† Supplied by Dr. S. A. Peoples and associates, School of Veterinary Medicine, University of California, Davis.

ever, to explain the involvement of 2 untreated first-lactation animals, 1 of which showed infection within a week of calving.

The milk procedure included prewashing of udders with a freshly prepared chlorine solution and dipping of teat cups in a similar solution after milking every second animal. The barn water was cultured on two occasions but Nocardia were not recovered.

Newborn calves were allowed to suckle their dams for 36 hours. The mouths of 10 calves, 3 to 21 days old, were swabbed, but Nocardia were not found.

Soil samples, from the holding corrals and cultivated fields, flies captured on cows and in the milking barn, dust from the barn rafters, dust from the flanks and udders of the cows, samples of feed concentrate, silage, and water from the corral trough were examined, by two methods, for Nocardia. Saline suspensions were made of the particulate material, and flies were shaken in a vial of saline solution. A portion of each preparation was injected into the peritoneal cavity of a guinea pig, and another portion was cultured by the method of Frey and Hagan.[10] The guinea pigs were killed after 14 days, and all lesions were cultured in serum broth and on tryptose agar plates.

Only three samples, those of the soil from each holding corral, yielded Nocardia. The organisms isolated were indistinguishable from those found in infected glands. Larger numbers of subcultures from each specimen probably would have yielded additional positive results. Isolations had been anticipated from the flanks and udder surfaces.

Fig. 10—Experimentally infected mammary gland, showing gross pathological changes. The fibrotic changes were pronounced in the gland to the right. The foci to the left of the intermammary septum have apparently penetrated into the noninjected gland.

Skin Testing with a Nocardial Extract and Tuberculin

In discussing the allergic properties of *N. asteroides*, Drake and Henrici [15] cite reports [9,28] of close antigenic relationship and cross-complement fixation between the tubercle bacillus and *N. asteroides*. They concluded that the skin sensitivity induced by oil suspensions of living *N. asteroides* inoculated intratesticularly did not cross react with tuberculin, nor did tuberculin-sensitive animals react to nocardial extracts which gave positive reactions in animals infected with *N. asteroides*. Freund and Lipton,[18] however, report some cross reaction in guinea pigs sensitized with *N. asteroides* emulsions and tested with tuberculin. Such a reaction might present a problem when dairy cattle are tested with tuberculin.

The cow experimentally infected with Nocardia was negative to tuberculin tests prior to, and 27 days after, inoculation. Six other animals from the diseased herd, which had shed *N. asteroides* in their milk on one or more occasions and had apparently been infected for one to two months, were negative to both caudal fold and cervical intradermal injections of tuberculin. Two had been successfully treated prior to the use of tuberculin and Nocardia organisms were not found in the milk of the remaining 4 on the day of tuberculin injection.

Extracts were made of the Nocardia isolated from the herd, corresponding approximately to the crude extract of Drake and Henrici.[15] One cow which showed no reaction to tuberculin subsequently reacted positively to this preparation; 4 other cows, 2 with and 2 without nocardial infection, showed no reactions.

Failure to isolate Nocardia from the selected "positive" animals on the day of tuberculin injection makes these results questionable, though 1 of these animals subsequently was shown to be sensitive to a nocardial antigen. However, until the converse is proved, tuberculin reactions resulting from *N. asteroides* infection in the mammary gland would seem improbable.

Discussion

In their discussion of the mycology of human nocardiosis, Conant *et al.*[10] state that there is no adequate taxonomic treatment of these aerobic actinomycetes due, in part, to incomplete descriptions of early isolation. For this reason, various aspects of this paper have been presented in considerable detail that it might better serve as a reference for future descriptions of the infection in the bovine mammary gland. The morphological, pathological, and biological characteristics of the isolated organism clearly fit the descriptions of *N. asteroides*. Antigenically, it has been found to agglutinate in high titer with *N. asteroides* antiserum. *Nocardia asteroides* is widely considered only "partially acid-fast," so the limited retention of carbol fuchsin by our isolates would not seem to justify a different name for the organism considered here.

During this investigation, another strain was submitted from a single quarter infection in south central California. The clinical description [7] of this case and the reactions of the isolate in our laboratory indicate that this must also be considered a bovine mammary gland infection of *N. asteroides*.

The colonies on blood agar plates have been described as simulating staphylococcic colonies.[27] Our experience indicates that such a resemblance may account for the paucity of reported udder infections because duplicate samples of milk, from which Nocardia organisms were isolated at this laboratory, were reported from another laboratory as micrococcic infections. It seems more likely that the infection is newly recognized than that it is a new udder resident.

Nocardial mastitis may have public health implications; the organism isolated in Canada survived more than 30 minutes at 60 to 70 C.,[2] and *Asteroides granulosus* isolated from milk in Italy survived pasteurization.[32] Our results also indicate that pasteurization may not inactivate the organism in infected milk. While we were unsuccessful in an attempt to infect a guinea pig by the oral route, there is still this possibility. Limited evidence indicates that *N. asteroides* infection of the bovine mammary gland does not engender tuberculin hypersensitivity; however, this possibility also merits more definitive exploration.

The pathological changes from the natural infection suggest, by their lobular distribution, an ascending infection. The presence of *N. asteroides* in the soil of all

three holding corrals makes this premise more probable.

Since the disease probably reaches the glandular parenchyma via the teat canal, disinfection of the teats with chlorine or benzalkonium chloride solutions at 200 p.p.m. after milking and before intramammary therapy is advisable. The infusion technique still is considered a possible means of spreading the infection; however, it fails to explain the infection in recently fresh, first-lactation heifers. Unusual virulence of the organism may be the reason for more frequent infections than have been seen previously. The earlier time of death in guinea pigs after intraperitoneal or intratesticular injection supports the assumption of unusual virulence.

The extensive pathological reaction with nocardial infection soon removes the gland from economic production. Bacteriological and clinical responses indicate that the described mixture of nitrofurazone and novobiocin has definite therapeutic value. The withdrawal of antibiotic therapy may, on occasion, be desirable;[3] however, when considering mycotic mammitis in general, the wisdom of excluding the aerobic actinomycetes from this rule-of-thumb is evident. In our experience, untreated animals soon became poor subjects for therapy and poorer producers of market milk. The only other successful treatment of nocardial *(farcinica)* mastitis we have encountered was surgical removal of the infected gland.[20]

SUMMARY

The organisms cultured from the milk of 28 cows (18%) in a dairy herd are described and identified as *Nocardia asteroides*. This infection has not been reported previously in this country, nor has it been shown to involve an appreciable segment of any herd.

The naturally infected cows showed extensive fibrosis and palpable granulomatous nodules in the infected mammary glands, altered milk with grossly visible white clumps of mycelia, and temperatures up to 107 F. A single fatality was observed.

Microscopically, the lesions showed extensive fibrosis, granuloma formation, and hypertrophic reaction of ductile and glandular epithelium. The lobular distribution of reaction indicated an ascending infection. Nocardia organisms were isolated from soil samples of all holding corrals on the infected premises.

The infectious agent has survived exposure to 64 C. for 30 minutes. Freshly prepared chlorine and benzalkonium chloride (Roccal) solutions at 200 p.p.m. were the most germicidal of the four commonly used disinfectants tested.

Tuberculin sensitivity was not demonstrated in animals considered clinically infected with *N. asteroides*.

A mixture of novobiocin (Albamycin) and nitrofurazone (Furacin) in 0.2 per cent solution was used successfully in infected udders.

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Blood Plasma Cholesterol Levels in Normal and Dwarf Beef Cattle

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SPORADIC dwarfism has occurred in man and in animals for centuries. Among animal breeders, a report by Craft and Orr,[7] in 1923, of an apparently cretinous dwarf beef calf was regarded as an interesting observation until dwarf conditions were described in the three major breeds of beef cattle by Johnson *et al.*,[10] Baker *et al.*,[2,3] Gregory *et al.*,[8] and others.

It is clear that more than one type of dwarfism exists in beef and dairy cattle, that there are anatomical and physiological variations in dwarfs of the same general type, and that the condition is heritable. The most common type of beef dwarf is small at birth, usually with a bulging forehead, and with some degree of hydrocephalus. The lower jaw usually protrudes beyond the upper dental pad, the tongue tends to protrude, the calves are often weak, and mortality is high. With increasing age, the small size becomes more apparent. The limbs are unusually short, a typical "pot-belly" develops, chronic bloating occurs, and breathing is noisy. Because of the labored breathing, many refer to this type of animal as the "snorter" dwarf. It was natural that comparisons would be made with human dwarfs, and many workers were struck by certain resemblances to the human hypothyroid cretin. Carroll *et al.*[6] were among the first to suggest that dwarf cattle were deficient in the pituitary thyrotropic hormone, although later work has shown that the condition is not due to abnormal thyroid function.

Because it was well known that hypothyroidism is associated with hypercholesterolemia in children (Wilkins *et al.*[19]), and that partial thyroidectomy is followed by an increase in blood cholesterol level in man (Hurxthal and Simpson[9]), it was decided to compare the blood cholesterol values of normal and dwarf cattle.

MATERIALS AND METHODS

A total of 175 determinations of blood plasma cholesterol levels were made on 22 male and 24 female dwarf calves representing three beef breeds, ranging in age from 11 to 580 days. Observations were made at all seasons of the year during a three-year period. Determinations (totaling 71) were also made on 42 normal cattle, predominantly of Shorthorn and Aberdeen Angus breeding. Blood plasma cholesterol levels were determined by a slight modification of the method of Pjoan and Walter.[16] The procedure is described in more detail in an accompanying paper by Means *et al.*[15]

All data were subjected to appropriate statistical analysis, including the analysis of variance and tests for the homogeneity of variance.

EXPERIMENTAL PROCEDURES AND RESULTS

The data on blood plasma cholesterol levels that were gathered from the dwarf animals over a three-year period were studied with respect to sex, age, and the season of the year. Data on normal beef cattle were obtained during December, January, and February, but under different temperature conditions which will be described.

The plasma cholesterol levels of normal nonpregnant females and of normal steers showed relatively little individual variation, the extreme range being 96.7 to 107.8 mg./100 ml. (table 1). There was no significant difference between heifers and steers. No studies were made of normal bulls.

When dwarfs less than 180 days old were compared with older dwarfs, no difference was found. A comparison of dwarf bulls and heifers showed a significantly greater level of blood cholesterol in the males. When all control animals and all dwarfs were compared, there was a highly significant difference (table 1). Both the mean values and ranges of blood plasma cholesterol levels were lower in the dwarfs. Since plasma cholesterol is usually increased in hypothyroidism, it seems apparent that

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TABLE 1—Blood Plasma Cholesterol Levels of Dwarf and Normal Beef Cattle

	No. Animals	No. determinations	Plasma cholesterol levels: Average (mg./100 ml.)	Plasma cholesterol levels: Range (mg./100 ml.)
Normal				
Steers	18	26	105.3 ± 3.1	102.8 — 107.8
Females	24	45	100.2 ± 2.7	96.7 — 103.7
Dwarfs				
Less than 180 days old	33	87	83.0 ± 5.2	76.2 — 90.8
More than 180 days old	20	88	79.9 ± 4.6	66.8 — 94.7
Bulls	22	81	89.4 ± 5.4 *	74.4 — 105.3
Heifers	24	94	73.5 ± 4.5	64.0 — 84.5
All normals	42	71	102.5 ± 2.0 †	99.4 — 105.5
All dwarfs	46	175	81.1 ± 3.6	68.9 — 94.4
Effect of season on dwarfs				
Dec. to Feb.	18	43	86.7 ± 8.3	79.9 — 94.7
Mar. to May	7	19	77.1 ± 4.0	72.4 — 82.5
June to Aug.	33	94	80.2 ± 3.8	71.2 — 90.4
Sept. to Nov.	2	18	64.0 ± 29.0	41.0 — 93.3

* Significantly higher than dwarf heifers ($P < 0.05$). † Significantly higher than all dwarf animals ($P < 0.01$).

this test of thyroid function does not indicate a state of hypothyroidism in the dwarf cattle.

With the exception of the September to November interval, during which only 2 dwarf animals were observed, there was little indication of a seasonal change in cholesterol levels. It is clear that there is no significant difference between the winter and summer periods (table 1). If thyroid activity is reduced during the summer, it would be expected that plasma cholesterol levels would rise. In actuality, the cholesterol values were somewhat higher in the December to February interval than in June, July, and August.

Because a severe cold period occurred in January of 1954, it was decided to compare the plasma cholesterol levels of normal and dwarf cattle prior to and following the temperature drop. Cold is regarded as an effective stress condition, and because of the known relationship between thyroid function and environmental temperature, this natural environmental change was regarded as being of possible significance.

Hereford dwarf calves, 4 male and 4 female, were compared with 10 normal Shorthorn and 10 normal Aberdeen Angus calves of both sexes (table 2). The dwarfs ranged from 203 to 756 days old and the control animals from 113 to 313 days old. Blood samples had been taken from both groups of animals on December 30 and 31. December temperatures had been unusually mild, ranging from 50 to 60 F. during the day and dropping to 25 to 30 F. at night. On January 10, the temperature dropped to —5 F. and varied from —5 to 10 F. for several days. Blood samples were taken again on January 25 and comparisons made of the effects of low temperatures on blood cholesterol levels. As shown (table 2), there was a reduction in blood cholesterol levels in both normal and dwarf cattle. The reduction was not statistically significant in the normal animals, but was significant in the dwarf animals ($P > 0.05$). Since it has been observed in other species that a reduction in environmental temperature is often followed by increased thyroid activity, it is interesting to speculate that the dwarf cattle responded to cold stress somewhat more effectively than the normal animals. This is regarded as additional evidence that the thyroid glands of dwarf cattle are relatively normal and not hypothyroid.

DISCUSSION

The blood plasma cholesterol levels observed in this study are comparable with those reported by other investigators. Albritton [1] reported a mean plasma cholesterol value of 110 mg./100 ml. in beef cattle. Terri *et al.*[18] found that, in dairy calves from 9 to 24 weeks of age, the range of plasma cholesterol levels was 71 to 186 mg./100 ml., and the mean value was 108 mg./100 ml. In blood samples taken from normal cattle at various seasons of the year, the average value was approximately 100 mg./100 ml. As previously mentioned, the plasma cholesterol values of the dwarfs were usually lower than those of the normal cattle.

TABLE 2—Blood Plasma Cholesterol Levels of Dwarf and Normal Beef Cattle Under Two Environmental Conditions

	No. animals	No. determinations	Plasma cholesterol levels: Average (mg./100 ml.)	Plasma cholesterol levels: Range (mg./100 ml.)
December 30 and 31				
Normal	20	20	101.8 ± 7.4	80.0 — 117.5
Dwarfs	8	8	114.8 ± 6.4	90.0 — 147.0
Janary 25				
Normal	20	20	94.3 ± 2.7	71.5 — 118.0
Dwarfs	8	8	97.0 ± 5.6	80.0 — 125.5
Both determinations				
Normal	20	40	98.0 ± 1.9	71.5 — 118.0
Dwarfs	8	16	106.4 ± 4.7	80.0 — 147.0

Because medical clinicians frequently use blood cholesterol levels as an index of thyroid activity, and because of the tendency of the thyroid to exhibit seasonal rhythmic activity, it had been anticipated that plasma cholesterol levels might vary in normal and dwarf cattle.

Brody [5] found that Jersey and Holstein-Friesian cows kept in a controlled climatic chamber showed a marked reduction in total blood cholesterol as ambient temperature was increased from 70 to 100 F. Blood cholesterol was highest during early lactation and declined with advancing lactation.

Long *et al.*[13] found that in dairy cattle the administration of thyroprotein produced a reduction of plasma cholesterol, followed by a gradual return to normal levels, and concluded that plasma cholesterol levels more accurately reflected the thyroid status of cows than blood protein bound iodine (PBI). Lewis and Ralston [12] found that, under farm conditions, PBI was lower in November than in the period from March through August. Blincoe *et al.*[4] found that, in a controlled environment, the PBI of dairy cows did not change over a temperature range of 7 to 105 F. These workers found, however, that when thyroid function was studied by appropriate radioiodine (I^{131}) techniques, the increase of ambient temperature to more than 60 to 95 F. caused a decrease in thyroid activity of 30 to 65 per cent in Holstein-Friesian, Jersey, Brown Swiss, and Brahman cattle, although Brahmans were least affected. A decrease of ambient temperature to below 32 to 17 F. increased thyroid activity from 60 to 100 per cent in Jersey and Brahman cattle, but not in the other two breeds. Over-all iodide metabolism, measured in terms of plasma iodide clearance, was not affected by ambient temperature. Lengemann *et al.*,[11] in a study of the concentration of I^{131} in the milk of Jersey cows, found that the onset of spring (March) was accompanied by increased I^{131} in blood and milk. They concluded that this finding supported the earlier report of Matthews *et al.*[14] that the iodine content of milk is lowest in late spring and highest in August, and the reports of Seiden *et al.*[17] that thyroidal iodine is highest from June to November and lowest from December to May.

SUMMARY

The blood plasma cholesterol levels of dwarf beef cattle were significantly lower than those of normal beef cattle. Since the cholesterol values of the sample of normal cattle used in these studies were in close agreement with published values for normal cattle, it was assumed that they were representative. The cholesterol levels of dwarf bulls were higher than those of dwarf heifers. There were no significant seasonal differences in blood plasma cholesterol levels of dwarf cattle; however, following a severe cold period, there was some reduction of blood cholesterol levels in both normal and dwarf cattle.

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Cerebrospinal Fluid Pressure in Dwarf and Normal Cattle

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DWARFISM HAS OCCURRED in domestic animals for many years but was regarded as a curiosity until it was described in the three major breeds of beef cattle by Johnson *et al.*,[5] Baker *et al.*,[1,2] Gregory *et al.*,[4] and others. It is recognized that more than one type of dwarfism exists in beef and dairy cattle, that there is some anatomical and physiological variation in dwarfs of the same general type, and that the condition is heritable. The most common type of dwarf beef cattle, which was studied in this investigation, shows some degree of bulging of the forehead and marked mismatching of the upper and lower jaws with pronounced protrusion of the mandible. Hydrocephalus of varying severity is usually present, and an increased amount of cerebrospinal fluid is usually apparent at necropsy.

Hydrocephalus, or increased cerebrospinal fluid pressure, or both, may result from several causes. As reviewed by Gilman,[3] congenital hydrocephalus occurs in man, cattle, swine, and various domestic and laboratory animals. Moore and Sykes[6] described an increase in the cerebrospinal fluid pressure of cattle with vitamin A deficiency and a similar finding was made in swine by Sorensen *et al.*[7] The desirability of comparing the cerebrospinal fluid pressures of normal and dwarf cattle is, therefore, apparent.

MATERIALS AND METHODS

Sykes and Moore[8] reported that spinal fluid pressures could not be measured by punctures in the lumbar region. They developed a technique for the penetration of the subarachnoid space through the dorsal opening of the atlanto-occipital articulation. The cerebrospinal fluid pressure was measured with the animal in a standing position, and without the use of anesthetics. It is necessary that the animals stand quietly throughout the entire procedure. Flexion of the atlanto-occipital articulation assists in penetrating the subarachnoid space. Undue struggling of the animal may, however, result in serious cord damage. In the dwarf animals, it was found that positive fluid pressures could be readily measured in the lumbar region by inserting a 100-mm., 17-gauge hypodermic needle, with stylet, into the subarachnoid space at the juncture of the last lumbar vertebra and the sacrum. If proper penetration was made, partial withdrawal of the stylet usually resulted in a show of fluid. When this occurred, a spinal fluid manometer of the water type was inserted into the needle (upon removal of the stylet) without the loss of fluid. With the manometer in the vertical position, spinal fluid was allowed to reach its own level and was recorded in millimeters. The animals were usually held loosely in a stanchion and no anesthetic was used. Minor fluctuation in fluid levels accompanied respiration, and struggling produced a sharp rise in fluid height. Measurements were made on a total of 56 dwarf beef animals representing the three major beef breeds. The animals were of both sexes and ranged from 11 to 580 days old. Comparisons were made with 27 dwarf-free purebred Shorthorn steers and heifers between 100 and 210 days old, and with 8 grade dairy calves from 1 to 2 years of age.

RESULTS AND DISCUSSION

The dwarf cattle exhibited high cerebrospinal fluid pressures at both the atlanto-occipital and lumbosacral locations (table 1). The pressures were highest in the occipital region. As previously reported by Sykes and Moore,[8] there was no significant pressure at the lumbosacral articulation in the control animals. The failure to obtain fluid pressures in the control animals raises questions regarding technique. In some of the control animals, spinal fluid rose slowly into the needle, but not under sufficient pressure to enter the manometer. When fluid could not be observed in the needle, a syringe was attached and a small amount of material was aspirated. Special care was exercised to explore several levels within the vertebral canal and to insure that the needle was not plugged. Because

TABLE 1—Cerebrospinal Fluid Pressures of Dwarf and Normal Cattle

Group	No. animals	Site of measurement	Av. pressure (mm.)	Range (mm.)
Dwarf	25	Atlanto-occipital	403	268-700
Dwarf	31	Lumbosacral	285	140-560
Normal Shorthorns	27	Lumbosacral	0	0-0
Normal dairy calves	8	Lumbosacral	0	0-0

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of the inherent dangers of measurement at the atlanto-occipital region, it was not possible to make direct comparisons of the two sites in the control and dwarf cattle.

In order to check the repeatability of measuring spinal fluid pressure, a number of measurements were made on 7 dwarfs at intervals ranging from several days to several months. Although there was considerable difference between dwarfs and between determinations, dwarfs tended to have consistently high cerebrospinal fluid pressures (table 2).

TABLE 2—Repeatability of Measuring Cerebrospinal Fluid Pressure in Dwarf Cattle at the Lumbosacral Joint

Repetition	Dwarf animal						
	1	2	3	4	5	6	7
1	170*	180	400	100	220	230	555
2	240	400	175	400	180	220	390
3	.....	280	100	230	210	236	380
4	.....	325	385	.....	.....	.....	.....
5	.....	240	.....	.....	.....	.....	.....

* Pressures are given in millimeters.

Because of failure to obtain positive cerebrospinal fluid pressures in 35 control animals, and because this was in agreement with the previous findings of Sykes and Moore,[8] it was decided to measure fluid pressures in a herd of cattle where dwarfism had occurred. A total of 53 calves ranging from 21 to 218 days old were inspected. All of these animals were of normal appearance and in apparent good health, and none of them were dwarfs. Their genetic makeup, with respect to the "dwarfing" gene, was unknown. It is likely that some of the animals were carriers of the dwarfing gene but, since proof is lacking, this can only be assumed. Of 53 calves measured, 29 had measurable fluid pressures at the lumbosacral joint and 24 did not have measurable pressures, although fluid entered the hypodermic needle in some of these cases. The pressures in some of these phenotypically normal animals were greater than in dwarf animals, ranging from 130 to 600 mm., but the animals showed no external signs of hydrocephalus. Observations were made from March through October. No seasonal trends were apparent and there was no clinical evidence of vitamin A deficiency.

Subsequent observations on a small number of animals known to carry the dwarfing gene, revealed that spinal fluid pressures may range from 0 to 500 mm.

Dwarf cattle of varying ages consistently exhibited high cerebrospinal fluid pressures. All of these animals showed external signs of hydrocephalus, and increased cerebrospinal fluid was evident at necropsy. The question of "normal" fluid pressure in "normal" cattle at the lumbosacral juncture is at present obscured. Measurements in presumably normal cattle at Purdue, which were believed to be free of the dwarfing gene, were in agreement with those of Sykes and Moore,[8] *i.e.*, that there is little, if any, positive pressure in the lumbosacral region. Observations in two herds of presumably mixed carrier and dwarf-free animals suggest that elevated spinal fluid pressures may occur in both types. The significance of this is unknown and warrants further research.

SUMMARY

Cerebrospinal fluid pressures were measured in a total of 56 dwarf beef cattle at the atlanto-occipital articulation or at the juncture of the last lumbar vertebra and the sacrum. Pressures ranged from 268 to 700 mm. at the atlanto-occipital region, and from 140 to 560 mm. in the lumbar region. In 35 normal beef or dairy cattle, presumably free of dwarfism, no positive pressure could be measured at the lumbosacral juncture. Of the 53 beef calves in a herd in which dwarf carriers could have been present, 29 exhibited positive pressures in the lumbar region, and 24 did not have measurable pressures. However, it does not appear that animals carrying the dwarf gene can be detected by the measurement of cerebrospinal fluid pressures.

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The Complement-Fixing Antibody Response of Cattle Infected or Exposed to Reinfection with *Dictyocaulus Viviparus*

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INVESTIGATIONS recently reported by Jarrett *et al.*,[3] Michel,[5,6] and Rubin and Lucker[7] have shown that cattle develop an effective acquired resistance or immunity to infection with the cattle lungworm, *Dictyocaulus viviparus*. Jarrett *et al.*[3] and Rubin and Weber[9] have shown that the administration of immune serum to susceptible calves confers a considerable degree of passive immunity to lungworm infection upon the recipient animals. Taylor[14] and Soliman[10] pointed out that the examination of feces for lungworm larvae is an unreliable method of diagnosis, and the former further stated that there is no present indication that diagnosis can be made by a sensitivity or agglutination test. Barker[1] believed that a protection could be set up against a respiratory disease, which he presumed to be parasitic bronchitis, by the employment of a bacterial vaccine isolated from the lung, whereas Stableforth[11] stated that much work has been done on allergic tests in parasitic bronchitis and that there was no doubt that a skin test and a complement-fixation response could be elicited.

Jarrett *et al.*[3] reported in their paper on passive immunization that circulating antibodies associated with *D. viviparus* infections are definitely detectable and measurable by a complement-fixation test. They used this test to measure the humoral antibody level in serum donors, in initially infected control calves, and in immunized test calves, and recorded the results in some detail for the animals of each category. The complement-fixation test that they employed was dependent upon the measurement of the amount of complement required to give 50 per cent hemolysis in the presence of immune serum plus antigen, and upon that required to give the same reaction in the presence of immune serum alone. The former amount divided by the latter was used to measure the potency of the serum. They did not describe the test fully, but Mulligan, one of the co-authors, later (personal communication) provided some information concerning the details of the method and indicated that several points remained to be worked out in the procedure, at least as regards its application to lungworm infections in cattle.

The work described in this paper was undertaken to obtain further information on the possibilities and reliability of the complement-fixation test (1) for the diagnosis of lungworm infection in cattle, (2) for measurement of the potency of immune serum, and (3) for the interpretation of parasitological data.

MATERIALS AND METHODS

History of Experimental Animals.—The serum samples used in this investigation were obtained from 27 calves and older cattle (table 1). Known negative serum was obtained from 3 calves (21, 49, and 80) which were never exposed to lungworm infection; from 4 cattle (3583, 3586, 3668, and 3670) which were maintained at the Animal Disease Station, Beltsville, Md., under conditions precluding exposure to the cattle lungworm; and from 7 calves (20, 22, 23, 35, 76, 77 and 80a) which were later experimentally infected with *D. viviparus*. All serums were used individually, except those obtained from the second group, which were pooled as controls for the complement-fixation tests. Test serum was obtained from 20 infected animals, the 7 calves just mentioned after they were infected, and 13 others.

These animals were allotted to three major groups as follows:

Group 1.—In group 1 were 13 animals from which blood samples were taken during the course of, and (or) after recovery from, an initial infection. Calves 42 and 44 of this group had been given infective larvae plus immune serum and had survived an initial infection, as described by Rubin and Weber.[9]

Group 2.—This group was composed of 4 calves from which blood samples were taken after they had been re-exposed to infection once only.

Group 3.—Group 3 included 3 calves from which blood samples were taken after they were re-exposed to infection three, four, and six times, respectively, over a period of several months.

The frequency with which the various test cattle were bled, the interval between the administration of infective larvae and the taking of the first blood sample, and the duration of the period during which the blood samples were collected varied according to the objectives in view. These

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data are summarized or are evident for each animal and the results of the complement-fixation tests are recorded (tables 2, 3, 4).

Infective lungworm larvae obtained from the culture of feces containing first-stage larvae of *D. viviparus* by methods essentially similar to those described by Rubin and Lucker [5] were administered *per os* to the 20 test animals as shown (table 1).

Preparation of Antigen.—Some antigens were prepared from the eggs, first-, second-, and third-stage larvae, and from agamic and mature lungworms which had been removed from the lungs of cattle slaughtered *in extremis* or which had been dead only a few hours. The processes used included lyophilization, extraction by boiling, high-speed centrifugation, and extraction with physiological saline solution. Barbiturate, phosphate, and saline buffers were used to dilute the various antigens. These antigens were tested for their combining power in serial dilutions of serums from known lungworm-positive and lungworm-negative animals, and for anticomplementary activity by the addition of saline solution instead of serum. Antigens produced by lyophilization and extraction by boiling or by high-speed centrifugation, or which were suspended in barbiturate and phosphate buffers, were generally not satisfactory because they were sometimes anticomplementary. Only the antigen prepared from mature worms which were extracted with physiological saline solution gave consistent results.

This antigen was prepared as follows: The worms were removed from the lungs, washed in physiological saline solution, and placed on filter paper. While still active, they were dried and refrigerated at —20 C. To each gram of worm tissue, 3 ml. of physiological saline solution was added, and the mixture ground in a Ten Broeck grinder. Then, 1-ml. amounts of the suspension were pipetted into separate glass ampules, which were placed in an alcohol-dry ice bath for five to ten minutes and then sealed with an acetylene torch. After rapid freezing at approximately —40 C., the filled ampules were stored at —38 C. The antigen was found to remain stable for long periods at this temperature without the addition of a preservative. The optimal complement-fixation results were obtained at a final dilution of 1: 60.

Technique of Test.—The serums were inactivated at 56 C. for 30 minutes after dilution with an equal volume of saline solution, and a six-tube serial dilution was run on each sample. Pipetting 0.25 ml. of diluted, inactivated serum with a serological pipette and adding saline solution with a Cornwall syringe was the most rapid method for preparing the dilutions. To each of them, 0.25 ml. of antigen was added, followed by 0.5 ml. of 2 full units of complement. After incubation for one hour, 0.5 ml. of sensitized cells was added, and the tubes were incubated for another hour. A total of 1.5 ml. was contained in each tube. After the second incubation period, the tubes were centrifuged at 1,500 r.p.m. for ten minutes and the amount of hemolysis was determined by a measured amount of cells precipitated. Complete inhibition of hemolysis was the criterion of a 4-plus reaction, and this 100 per cent end point was used as the index to the titer in which complement was fixed. Overnight refrigeration caused a settling of cells comparable to that obtained with centrifugation, but in some cases the amount was not well defined.

As the tests progressed, a general trend in results became apparent, and it was usually possible to predetermine the suitable range for the type of infection and the interval after infection when the sample was collected. If none of the six tubes in the serial dilution showed a 4-plus reaction, another range, overlapping the upper or lower extremes of the first series, was run, giving a maximum range of 1: 2 to 1: 2,048.

RESULTS

Response to Initial Infection and to Initial Infection Plus One Re-exposure.—Calves 22, 23, and 76, given initial infections only, and calves 46, 47, and 48, given initial infections plus one re-exposure to infection with *D. viviparus*, were bled almost daily for 43 to 70 days after the administration of the larvae. The results of tests on the serums of these animals (table 2) indicated that there was essentially no difference between the two groups in complement-fixing antibody response during this period. The intervals from the administration of the larvae to the initial rise in titer, and from this rise to attainment of the peak of the titer curve, as well as the total of these periods, were strikingly similar. The levels of the plateau titers and the duration of the plateau period, the latter determinable for only 5 of the calves because calf 76 died prematurely, also were not materially different in the two groups, except that the titer for re-exposed calf 46 was consistently low.

Although 3 calves of the group which was re-exposed once had been initially infected with relatively small doses of larvae (table 1), it is surprising that the plateau titers (table 3) for none of them appreciably exceeded the average for the initially infected group. The low titer observed in calf 46 was consistent with data, not fully shown here, which indicated its initial great susceptibility to lungworm infection and its inability to develop a strong resistance to reinfection.

Range in Plateau Titer.—The range in the titers for these 6 calves and for 5

initially infected calves that were bled at comparatively infrequent intervals during the plateau period are shown (table 3). Although no samples were run from the latter group of calves prior to 19 days after infection, the almost daily tests on the first 6 calves had shown that the peak titer was unlikely to occur prior to that time.

The only animals for which the mean plateau titers fell outside of the limits 1:128 to 1:512 were calf 35 (1:64), which died only 42 days after infection, and calf 46 (1:8), the exceptional behavior of which has been previously discussed. It is also notable that the mean titers for initially infected calves 100, 101, and 102 were higher than the mean for any of the 3 calves which had been re-exposed once.

Although there was some variation in the titers for the individual animals during the plateau period, the majority of the samples fell within one serial dilution of the mean titer. The titer for a few of the animals, as for example calf 47, showed wide variation from the mean but was constant in closely consecutive samples obtained at approximately the same interval after exposure to infection. Day-to-day variations were sometimes encountered in the positive controls, and when this occurred the samples of unknown titer were run again, but for the most part the results were reproducible.

Relation of Titer to Parasitological and Other Data.—As indicated by the complement-fixation titers, the antibody response of the 8 initially infected calves (table 3) was not related to the size of the infecting dose of larvae. For example, the mean plateau titers were higher for calves

TABLE 1—Parasitological History of Calves from Which Serum Samples Were Obtained

Animal No.	Initial infection: Age of calf (mo.)	Initial infection: No. larvae administered	Initial infection: Duration of patent period (days)	Re-exposure: Age of calf (mo.)	Re-exposure: No. larvae administered	First-stage larvae recovered from feces (total No.) #
			CONTROLS			
21	Not infected		0	Not infected		0
49	Not infected		0	Not infected		0
80	Not infected		0	Not infected		0
3583*	Not infected		0	Not infected		0
3586*	Not infected		0	Not infected		0
3668*	Not infected		0	Not infected		0
3670*	Not infected		0	Not infected		0
			INITIAL INFECTION			
20**	6.9	7,500	16	Not infected		†
22**	7.0	25,000	31	Not infected		2,530,000
23**	7.5	25,000	34	Not infected		15,440,000
26	6.1	7,500	19	Not infected		†
35**	4.1	10,000	‡	Not infected		†
42§	1.2	50,000	43	Not infected		2,111,000
44§	1.2	50,000	43	Not infected		947,000
76**	5.9	13,000	‡	Not infected		†
77**	4.5	8,000	49	Not infected		†
80a**	3.5	12,000	19	Not infected		†
100	2.0	5,000	28	Not infected		1,800,000
101	2.0	5,000	25	Not infected		1,760,000
102	2.0	5,000	27	Not infected		4,100,000
			ONE RE-EXPOSURE			
46	0.3	1,500	93	6.5	25,000	6,740,000
47	0.3	500	54	5.9	25,000	2,220,000
48	0.4	500	88	7.0	25,000	936,000
86	0.5	15,000	50	9.9	64,000	†
			MULTIPLE RE-EXPOSURE ‖			
61	1.4	10,000	16	8—12(3)	825,000	†
93	0.8	5,000	74	19—22(4)	1,423,000	2,727,000
96	2.5	20,000	35	18—21(6)	1,781,000	15,042,000

During initial infection only. * Cattle from lungworm-free herd at Animal Disease Station. ** Source of control serum before infection. † Not estimated. ‡ Died before end of patent period. § Also given immune serum as described by Rubin and Weber.[9] ‖ Number of re-exposures in parentheses. Doses, given at intervals over a period of several months, ranged from 25,000 to 750,000 larvae.

TABLE 2—Results of Complement-Fixation Tests on Serums of Calves Initially Infected and Re-exposed Once to Infection with D. Viviparus—Early Responses

Animal No.	Sampling period: Days	Sampling period: No. of serum samples	Time interval from—: exposure to intial rise in titer (days)	Time interval from—: initial rise to plateau titer (days)	Time interval from—: exposure to plateau titer	Duration of plateau titer (days)	Plateau titer
			INITIAL INFECTION				
22	92	60	12	6	18	46	1:128
23	65	60	12	6	18	49	1:256
76 *	42	43	14	2	16	.... *	1:128
Average for initial exposures			12.6	4.6	17.3	47.5	....
			ONE RE-EXPOSURE				
46	65	60	13	3	16	42	1:8
47	89	61	10	2	12	58	1:256
48	68	64	13	4	17	48	1:256
Average for re-exposures			12.0	3.0	15.0	49.3	....
Average for both groups			12.3	3.8	16.5	48.6	....

* Died 43 days after infection.

100, 101, and 102 that were given 5,000 larvae, than for calves 22 and 23 that were given 25,000. Also, the amount of complement-fixing antibody during the peak period of the rise did not show any correlation with the age of the calf at the time of infection. The titer was highest for the youngest of the group, next highest for the oldest, and lowest for the ones intermediate in age. No relationship was apparent between the titers and the severity or type of clinical signs shown by the calves. During the plateau period, the serum titers for calves 35, 76, and 102, that, as data not fully recorded here showed, were severely affected by their infections (2 of them fatally), ranged through four dilutions from 1:64 to 1:512, whereas the titers for calves 22, 23, 77, 100, and 101, all of which had mild signs, ranged over the same limits, the lowest dilution excepted. There was also no correlation between the titers and total output of first-stage larvae in the feces during the period of infection in 5 of these 8 calves for which the output was determined (table 1).

Persistence of Antibodies; Response to Repeated Exposures.—At various intervals (table 4) after the end of the plateau period, *i.e.*, nine weeks or more after an administration of larvae, samples of serum were obtained from calves 22, 23, 46, 47, and 48 (previously mentioned) and from 9 other experimental cattle, 3 of which had been repeatedly re-exposed to infection. Tests on these samples showed that complement-fixing antibodies usually were still present in the blood many weeks to several months after initial infection or a

TABLE 3—Results of Complement-Fixation Tests on Serums of Calves Initially Infected and Re-exposed Once to Infection with D. Viviparus—Titer Ranges During Plateau Period

Animal No.	Sampling period days after last exposure	No. of samples	Titers: 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
			INITIAL INFECTION									
22	18-64	44	....	....	....	....	....	10	28 *	6	....	....
23	18-67	28	....	....	....	....	....	3	4	10	8	3
35	20-41	8	....	....	....	....	3	5	....	....	....	....
76	17-43	27	....	....	....	....	....	3	15	9	....	....
77	19-34	7	....	....	....	....	....	2	5	....	....	....
100	21-67	8	....	....	....	....	....	....	....	1	6	1
101	21-53	6	....	....	....	....	....	....	1	2	3	....
102	21-53	6	....	....	....	....	....	....	....	....	5	1
			ONE RE-EXPOSURE									
46	16-58	28	2	11	7	5	3	....	....	....	....	....
47	12-71	61**	....	....	....	....	4	12	14	23	6	2
48	17-64	34	....	....	....	....	....	3	5	18	8	....

* Mean titer of each animal underlined. ** Large number of samples run because of variations in titers.

TABLE 4—Results of Complement-Fixation Test on Serums of Cattle Infected with D. Viviparus After Initial Infection and Single and Multiple Re-exposures—Titer Ranges in the Postplateau Period

Animal No.	Sampling period weeks after last exposure	No. of samples	Titers									
			0	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
INITIAL INFECTION												
42 *	24-49	6	3	1	2	....	....	....	....	....	....	....
44 *	22-50	7	....	4	3	....	....	....	....	....	....	....
22	39-40	4	....	....	....	4	....	....	....	....	....	....
23	34-36	4	....	....	....	....	3	1	....	....	....	....
26	21-32	4	....	....	....	....	2	1	1	....	....	....
20	21-52	4	....	....	....	....	....	....	3	1	....	....
80a	13-14	3	....	....	....	....	....	....	2	1	....	....
ONE RE-EXPOSURE												
46	9-36	4	3	....	....	1	....	....	....	....	....	....
48	25-28	8	....	....	....	5	3	....	....	....	....	....
47	39-41	3	....	....	....	....	....	3	....	....	....	....
86	28-80	4	....	....	....	....	....	....	1	3	....	....
MULTIPLE RE-EXPOSURE												
93	31-34	8	3	1	....	3	1	....	....	....	....	....
61	10-39	4	....	....	....	....	....	....	....	3	1	....
96	33-36	7	....	....	....	....	....	....		1	5	1

* These animals received larvae and immune serum.

re-exposure to infection with *D. viviparus*. Although the titers of only a few samples from each animal were ascertained, the results for the majority of the serums were remarkably consistent. Where samples from a particular animal were distributed over a period of a month or less, the fact that the titer usually did not vary more than one serial dilution from the mean was significant, mainly as evidence of the reproducibility of results with the method of testing used. However, blood samples were collected from some animals over a long period (table 4) to permit the determination of the stability of the titer.

In the postplateau period, at the intervals after initial infection or exposure to reinfection tested, the titers were, in general, lowest (mean 1:2 to 1:4) for the 2 animals that were given immune serum plus one dose of infective larvae, intermediate (mean 1:16 to 1:64) for the other initially infected animals and for those re-exposed once to infection, and highest (mean 1:128 to 1:256) for those that had been re-exposed to infection repeatedly (table 4). At present, no explanation can be given for the fact that all samples from animal 93 of the last-mentioned group showed either complete hemolysis at any dilution or low titers. This animal was undoubtedly highly resistant to reinfection. It was given a total of almost 1.5 million larvae, in several widely spaced challenge doses. No clinical effects were evident after any of these administrations; yet the complement-fixation titer of the animal's serum did not indicate resistance.

Other tests on appropriately timed samples appeared to show that the titers of the serums of animals 61, 93, and 96, whose resistance was repeatedly rechallenged and found to be highly effective, did not change as the number of re-exposures to which they were subjected was increased, and remained about the same whether the number of larvae used for a particular rechallenge was relatively small or relatively large (table 1).

Unfortunately, the plateau titers, *i.e.*, the mean titers during the period from about two and one half to nine weeks after the administration of infective larvae, had been determined (tables 2 and 3) for only 5 of the animals listed in table 4, 2 of which (22 and 23) had been initially infected only, and 3 (46, 47, and 48) re-exposed once to infection. A diminution in titers with the passage of time was demonstrated in these animals.

However, in the postplateau period, the titers for several animals, notably but not exclusively those that had been repeatedly re-exposed to infection, were found to persist at about the same levels for several months in the absence of restimulation of antibody production. Especially notable was the fact that the titer of the serum of calf 86 which had been re-exposed once persisted at about the same level for one year.

DISCUSSION

Using as the measure of increase in titer the expression

$$\frac{\text{immune serum + antigen}}{\text{immune serum}},$$

previously mentioned, Jarrett *et al.*[3] found, in their experiment on passive immunization, that the titers of the serums of calves initially infected as controls in every case progressively increased from the third to the thirtieth day of infection. The titers of the serums of the 3 initially infected and frequently bled calves in the present series presented a somewhat different picture during the first postinfection month in that no response was detectable prior to the twelfth day and peak titers were rapidly attained about five days later. Although Jarrett *et al.*[3] did not specify the exact level of the titer of the serum they obtained from their serum donors, it evidently was high. However, the writer found essentially no difference in serum titers between initially infected calves and calves re-exposed once to infection; but it must be re-emphasized that the calves which were re-exposed once had been initially infected with small numbers of larvae; therefore, their antbiody response on re-exposure to infection may have been atypical. Titers in the serums of some initially infected animals were as high as in the serums of any repeatedly re-exposed animal tested. However, although the writer's findings seem to show that booster doses of larvae had no appreciable effect in increasing the antibody titers of the serums of animals already rendered immune to *D. viviparus* by several prior exposures to infection, no data are available to show how high the serum titers of these individuals may have been before they were first tested. Under experimental conditions, the maximum response may well ordinarily be elicited by the second or third exposure to which an animal is subjected.

Stewart[12] found that the serological response of older sheep to infestation with Trichostrongylus spp. was earlier and more vigorous than that of younger sheep. However, the writer found no correlation between serum titers and ages of calves that were initially infected with *D. viviparus* when from 2.0 to 7.5 months old, but observations were not made on any appreciably older initially infected animals. Stewart[13] also reported that established infections with mature *Haemonchus contortus* in sheep elicited little or no complement-fixing response and that serums from sheep dying, presumably with a high level of infection, were negative to the test. He[12] likewise found that the serums of sheep dying from infection with Trichostrongylus spp. were negative to the test, although, in contrast to the findings for Haemonchus, infections with adult Trichostrongylus were found to stimulate antibody production. In the present investigation, the serum titers of 2 calves that died of lungworm disease while their lungs still contained numerous worms were found to be still near the peak level at the time of death. Also, in two cases studied especially to determine whether the duration of the patent period of the infection and the duration of the plateau titers were correlated, the titers did not decline until the infection was about to terminate or had terminated.

In the course of initial *D. viviparus* infections, larval worms are found in the lungs as early as the third day of infection,[10] gross respiratory signs appear as early as the seventh[7] to the twelfth day,[4] and young adults are in the bronchi on the eighteenth day.[2] In the present tests, the initial rise in complement-fixation titers was shown to occur at the time Jarrett *et al.* gave for the earliest appearance of gross respiratory signs, and the average time required for the complement-fixing titers to reach a peak after initial exposure coincided with the time when the worms are reported to reach young adulthood in the bronchi. Hence, it seems that the presence of the larval worms *per se* was not sufficient to elicit the antibody response. Since the worms are lying free in the air passages of the lungs, it could well be the toxins produced by them that finally bring about the response. Rubin and Lucker[7] found that, although some of the larvae administered reached the lungs of resistant animals, nearly all of the young worms usually were eliminated in an immature state within three weeks. The writer's findings on initially re-exposed calves indicate that the maximum complement-fixing antibody response is attained prior to this time.

Jarrett *et al.*[3] obtained immune serum from animals whose antibody titers had been boosted by "experimental reinfection" with doses ranging from 50,000 to

200,000 infective larvae of *D. viviparus*. These animals showed a typical "secondary response," the level of complement-fixing antibody reaching a maximum two to four weeks after the experimental infection. In the 3 once re-exposed calves bled almost daily in the present experiment, the peak of the serum titer "secondary response" came 12 to 17 days after administration of the challenge dose of larvae.

Jarrett *et al.*[3] found that increased resistance to reinfection is accompanied by a rise in the titer of complement-fixing antibodies in the serum, whereas the writer found no essential difference in the maximal serum titers of initially infected, once re-exposed, and repeatedly re-exposed groups of animals. However, certain qualifications as regards the findings for the latter two groups have already been mentioned, and the persistence of the titers at high levels was maximal in 2 of the 3 animals of the repeatedly exposed group. The persistently low titer of the third member of this immune group has been mentioned; and an experiment is planned to resolve the question whether serum from this animal has strong protective power, despite its low complement-fixing antibody titer.

SUMMARY

Complement-fixation tests were made on the serums of 27 calves and older cattle, 20 of which were exposed to *Dictyocaulus viviparus* infection. The complement-fixation responses of 3 calves initially exposed, and of 3 calves initially infected and once re-exposed to infection, were not significantly different. The time from initial infection or re-exposure to the initial rise in titer averaged slightly over 12 days (12.6 vs. 12.0), and the interval from the initial rise to the peak of the response averaged about four (4.6 vs. 3.0) additional days. The duration of the period in which the titers remained near the peak level was about 48 days in both groups. The range of the complement-fixation titers of serums from these calves and from 5 other initially exposed calves during this period was found, as a rule, to be within the limits of a three-tube dilution. Nine weeks, or longer, after being given infective larvae, animals which had been passively immunized showed the lowest antibody response; repeatedly challenged animals showed the greatest response; other animals initially infected, and those initially infected and re-exposed once to infection, showed intermediate titers. There seemed to be no relationship between the complement-fixing antibody responses of initially infected calves, their ages at the time of infection, the number of larvae administered to them, the number of first-stage larvae subsequently eliminated in their feces, or the severity of the effects of the infection.

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The Pharmacology of Sulfabromomethazine, a New Long-Acting Sulfonamide, in Cattle

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THE VIEW has long been held that it is desirable, if not virtually essential, to establish and maintain a constant blood level of a sulfonamide throughout a course of therapy. The magnitude of this blood concentration has not been determined conclusively for various infections, but, in man, blood levels of 8 to 15 mg./100 ml. are considered desirable.

Maclay and Slavin[9] have suggested that 5 mg./100 ml. be considered the approximate minimum effective therapeutic blood level in animals. This value has become more or less generally accepted in veterinary circles. In so far as blood levels are concerned, these usually are unimportant, per se, except when an active bacteremia exists. However, there is a great body of information which indicates that the sulfonamide blood level is an index of the tissue level, as well as the total amount of sulfonamide in the body. This general view has developed from the work of Marshall and coworkers, and is pointed out in Marshall's review[10] of the dosage schedule of chemotherapeutic agents.

Since the "constant" level concept of sulfonamide therapy has been generally accepted, the problem of maintaining effective blood levels after single doses in cattle has received considerable attention. The older group of sulfonamides, such as sulfathiazole, sulfapyridine, and the methylated sulfapyrimidines will maintain "therapeutic" concentrations for six to 24 hours after single intravenous doses of 143 to 214 mg. per kilogram (1.0 to 1.5 gr./lb.). Consequently, veterinarians using the most advantageous sulfonamide (sulfamethazine) still must re-treat their patients once a day. If, however, sulfonamides were available that maintained therapeutic blood levels for two days or longer after single doses, a step forward would have been made.

Preliminary investigations by McManus *et al.*[11] with certain halogenated sulfapyrimidines showed that introduction of a chlorine or bromine atom into the number five position of the pyrimidine nucleus of sulfamerazine resulted in a prolongation of the plasma levels. Furthermore, the bromo derivatives were shown by Waletsky and Hughes[16] to be some ten times more effective than sulfaguanidine against avian coccidiosis, while English *et al.*[5] found that sulfabromodiazine, sulfabromomerazine, and sulfabromomethazine displayed *in vitro* and *in vivo* activity of the same order of magnitude as sulfathiazole and sulfadiazine. Furthermore, Miller[18] stated that the brominated compounds exhibited approximately the same degree of activity as the other sulfonamides in current use.

In view of these findings, the possibility of using sulfabromomethazine and sulfabromomerazine as systemic sulfonamides in cattle and other domestic species presented itself. If further investigations revealed that, with suitable doses, therapeutic blood levels could be maintained for two days or longer after single doses, it was felt that there would be a wide field of usefulness for such a drug. It was with these specific aims and purposes in mind that these studies were undertaken.

MATERIALS AND METHODS

All sulfonamide determinations were done by the method of Bratton and Marshall, by comparing the color intensity of unknown blood, plasma, milk, cerebrospinal fluid, and urine samples with standard solutions prepared at concentrations of 2.5, 5.0, 10.0, and 20.0 mg./100 ml. A Klett-Summerson photoelectric colorimeter (green filter, 540 mμ) was used to measure optical density. Standard solutions for blood and plasma were prepared by adding the requisite amounts of sulfabromomethazine to pooled blood and plasma that were obtained from each of the animals before the drug was given. Recovery of the drug from whole blood and plasma was found to average 73 and 91 per cent, respectively. With milk, cerebrospinal fluid, and urine, recovery was virtually 100 per cent and, hence, water standards were used in assaying these particular body fluids.

The assay of sulfabromomethazine is unique because the color that is developed after the addition of the coupling reagent (N-1-naphthylethylenediamine dihydro-chloride) fades rapidly for about five to ten minutes, and then becomes relatively stable. This decrease in color intensity with time was quantitated, and it was found that, if optical densities were measured exactly ten minutes

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after addition of the coupling reagent, error was minimized. A substantial error would be introduced if optical densities were not measured at a constant time interval after completion of the chemical reaction. The solubility of sulfabromomethazine was determined by the method originally described by Lehr.[8]

The apparent volume of distribution was estimated by the method used for determinations of various body fluid spaces.[4]

Cerebrospinal fluid was obtained by careful puncture through the foramen magnum. Only clear spinal fluid was used. If a sample became contaminated with blood, it was rejected.

Kidney excretion of the drug was measured by placing calves in a metal metabolism cage and collecting 24-hour urine samples.

Bromide ion determinations were made on blood filtrates, using the gold chloride method of Wuth.[16]

All animals used for these experiments were clinically healthy cows, heifers, and calves. The majority consisted of grade Holstein-Friesian stock.

RESULTS

The results of the solubility determinations (table 1), and the whole blood and plasma levels of free sulfonamide after oral, intravenous, and intraperitoneal doses of sulfabromomethazine (tables 2-6), are shown.

TABLE 1—Solubility of Brominated Sulfapyrimidines

pH	Sulfa-bromo-methazine	Sulfa-bromo-merazine	Sulfa-methazine	Sulfa-merazine
5.5	0.4	0.7	56	34
6.5	1.9	4.5	60	55
7.3	7.3	13.6	99	143
8.0	45.0	50.0	228	264

All values are in milligrams per 100 ml., at 37 C.

The higher blood levels obtained with sulfabromomethazine, as compared with sulfamethazine, indicated that the bromine atom alters some body function so that drug retention was favored. In an effort to shed some light on the possible mechanisms involved, it was decided to administer sulfamethazine, together with stoichiometrically equivalent doses of bromide. The results of this experiment (table 7) show that the prolonged blood levels obtained with sulfabromomethazine are not due to the action of free bromide ions; rather, they must be ascribed to the intact sulfabromomethazine molecule itself. Furthermore, free bromide was not detected in the blood of calves treated with sulfabromomethazine.

Plasma Levels of Sulfabromomethazine.—Plasma levels and whole blood levels were determined concurrently after oral administration of the drug. The plasma concentrations were somewhat higher than the corresponding whole blood concentrations, usually by a factor of approximately 10 per cent.

Apparent Volume of Distribution.—From a knowledge of plasma concentrations of a drug, it is possible to estimate the apparent volume of distribution. Although it is recognized that there are certain assumptions implicit in a theoretical concept such as this, it is nevertheless felt to be sufficiently worthwhile to use such information as an approximation of the degree of tissue penetration. Most sulfonamides have an apparent volume of distribution of 35 to 60 per cent of body weight. The method of calculation is shown (table 8). These data suggest that sulfabromomethazine penetrates intracellular water to a limited extent.

Acetylation of Sulfabromomethazine.—The total sulfonamide concentration was determined after acid hydrolysis by the method of Bratton and Marshall. This procedure gives the total sulfonamide concentration and, by deducting the free sulfonamide concentration from it, the amount

TABLE 2—Whole Blood Concentrations of Sulfabromomethazine*

Time (hours)	Calves										Average
	1273	1274	1283	1319	1329	1342	1346	1347	1349	1350	
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	0.3	1.2	0.9	....	....	....	....	....	....	....	0.8
2	1.0	3.0	2.2	1.8	1.4	1.9	2.3	1.7	1.2	2.1	1.9
4	3.5	5.4	6.0	2.5	2.1	2.9	3.4	2.5	3.4	4.9	3.7
8	9.1	7.8	10.1	6.6	5.0	6.1	7.8	7.3	8.3	9.4	7.8
12	9.6	7.6	10.7	8.3	5.8	7.5	8.7	8.2	....	10.6	8.6
24	11.5	5.8	10.2	9.2	5.9	8.4	10.8	8.4	8.6	8.7	8.8
36	8.2	4.1	9.5	5.2	3.9	5.7	9.1	5.5	6.0	7.1	6.4
48	5.4	1.7	7.7	3.6	3.2	4.8	7.1	4.4	3.4	5.2	4.7
60	4.9	1.5	5.7	0.3	1.1	2.2	3.6	2.4	0.4	1.9	2.4
72	3.5	1.2	2.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.8

* Given orally at doses of 1.5 gr. per pound (214 mg./kg.).
All values are in milligrams per 100 ml. of whole blood.

TABLE 3—Plasma Levels of Sulfabromomethazine* in Calves

Time (hours)	Calves										Average	Range
	1273	1274	1283	1319	1329	1342	1346	1347	1349	1350		
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0– 0.0
1	0.3	1.2	0.9	....	....	....	....	....	....	....	0.8	....
2	1.0	3.0	2.2	....	....	....	....	....	....	....	2.1	....
4	3.5	5.4	6.0	....	....	....	....	....	....	....	5.0	....
8	9.1	7.8	10.1	7.8	5.0	7.5	8.2	9.1	9.6	10.9	8.5	5.0–10.9
12	9.6	7.6	10.7	9.2	6.1	7.9	7.9	9.4	9.9	11.4	9.0	6.1–11.4
24	11.5	5.8	10.2	9.4	7.7	8.7	11.4	10.9	10.8	11.5	9.8	5.8–11.5
36	8.2	4.3	9.5	7.2	5.2	7.9	10.0	8.2	8.2	9.2	7.8	4.3–10.0
48	5.1	1.8	7.6	4.0	3.2	5.5	7.8	....	4.6	6.3	5.1	1.8– 7.8
60	1.8	0.0	5.6	1.0	1.9	3.1	4.0	3.7	1.2	2.3	2.5	0.0– 5.6
72	0.0	0.0	....	0.5	1.0	1.5	1.4	1.8	0.6	0.6	0.7	0.0– 1.8

* Given orally at doses of 1.5 gr. per pound (214 mg./kg.)
All values are in milligrams per 100 ml. of plasma.

of drug that has been acetylated is obtained. Under these conditions, approximately 12 per cent of the total sulfonamide present in the blood of calves, after treatment with sulfabromomethazine, is present in the acetylated form. This is a low degree of acetylation, as compared to that obtained with other sulfonamides.

TABLE 4—Whole Blood Levels of Sulfabromomethazine*

Time (hours)	Calves			
	1365 (3 gr./lb.)	1366 (3 gr./lb.)	1351 (6 gr./lb.)	1352 (6 gr./lb.)
0	0.0	0.0	0.0	0.0
12	7.4	8.1	10.3	11.3
24	10.0	9.2	9.7	12.1
36	10.3	11.8	8.0	12.5
48	8.8	9.2	4.6	10.8

* Given orally at 3 and 6 gr. per pound (429 and 858 mg./kg., respectively).
All values are in milligrams per 100 ml. of whole blood.

Milk Levels of Sulfabromomethazine.— The milk concentrations in cows after oral administration of 1.50 gr. per pound, and intravenous administration of 0.94 gr. per pound are shown (table 9). The milk levels in the case of oral administration were low and did not exceed 1.7 mg./100 ml. The sulfonamide penetrates the mammary gland quickly, but shows some lag when the blood levels decline. The over-all average ratio of milk levels to blood levels was 0.2 to 0.25.

When given intravenously, the drug penetrated the udder quickly, and concentrations of 4 to 5 mg./100 ml. were attained during the period of peak blood levels. As with oral administration, there was a lag of about 12 hours during which the drug was detectable in milk, but not in blood. The over-all average ratio of milk to blood concentrations was approximately 0.2.

Cerebrospinal Fluid Levels of Sulfabromomethazine. — Cerebrospinal fluid was drawn from the foramen magnum and subjected to the standard procedure, using aqueous sulfonamide standards for comparison. Only clear, colorless samples of cerebrospinal fluid were used. The relationship between cerebrospinal fluid concentrations and plasma concentrations in calves is shown (table 10). Although the drug does penetrate the blood-brain barrier, it does so in low concentrations. The ratio of cere-

TABLE 5—Whole Blood Levels of Sulfabromomethazine* in Calves

Time (hours post-administration)	Calves							Average	Range
	1143	1164	1165	1237	1239	1398	1423		
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	.....
1	23.4	19.9	26.3	I†	I	38.4	38.9	29.4	19.9–38.9
2	22.9	19.7	25.5	I	I	33.9	38.7	28.1	19.7–38.7
4	21.8	19.3	23.9	I	I	33.1	.....	24.5	19.3–33.1
6	19.5	18.2	23.1	I	I	30.4	34.4	25.1	18.2–34.4
9	16.8	16.4	21.4	I	I	27.6	34.0	23.2	16.4–34.0
12	15.5	14.5	18.7	18.2	15.7	25.5	30.4	19.8	14.5–30.4
24	11.2	9.5	13.4	8.1	6.8	19.0	25.5	13.4	6.8–25.5
36	8.7	5.4	7.7	3.2	2.3	14.6	21.7	9.1	2.3–21.7
48	2.4	1.2	4.1	1.2	0.3	11.3	17.7	5.5	0.3–17.7
60	0.5	0.0	0.5	0.5	0.1	10.4	14.8	3.8	0.0–14.8
72	0.0	0.0	0.0	0.0	0.0	9.5	11.3	3.0	0.0–11.3

* Given intravenously at a dose of 1.5 gr. per pound (214 mg./kg.); †I = indeterminate value.
All values are in milligrams per 100 ml. of whole blood.

TABLE 6—Blood Levels of Sulfabromomethazine* in Cows

Time (hours post-administration)	Cows			Average	Range
	1575	1576	1580		
0	0.0	0.0	0.0	0.0	.....
1	20.1	11.0	4.9	12.0	4.9–20.1
2	21.2	14.4	7.7	14.4	7.7–21.2
4	20.1	15.5	12.8	16.1	12.8–20.1
8	17.8	12.4	15.4	15.2	12.4–17.8
12	14.1	9.7	14.9	12.9	9.7–14.9
17	14.5	7.8	12.2	11.5	7.8–14.5
27	12.0	5.6	8.4	8.7	5.6–12.0
38	10.1	5.1	5.1	6.8	5.1–10.1
49.5	6.4	3.4	3.2	4.3	3.2– 6.4
61	3.1	2.9	3.1	3.0	2.9– 3.1
74	0.4	0.1	0.2	0.2	0.1– 0.4

* Given intraperitoneally at a dose of 1.5 gr. per pound (214 mg./kg.).
All values are in milligrams per 100 ml. of whole blood.

TABLE 7—Whole Blood Levels of Sulfamethazine in Calves

Time (hours)	Calves					
	1325		1348		1349	1350
	SMT* + NaBr†	SMT*	SMT* + NaBr†	SMT*	SMT*	SMT*
0	0.0	0.0	0.0	0.0	0.0	0.0
12	3.5	16.9	2.0	15.2	17.7	17.0
24	1.5	8.1	3.5	8.1	11.6	11.7
36	0.0	3.0	6.0	3.4	6.7	8.5
48	1.5	0.7	3.0	0.7	3.4	4.2
60	0.0	0.0	2.5	0.0	2.3	2.7

* SMT = sulfamethazine, given orally at a dose of 214 mg. per kilogram; † NaBr = sodium bromide, given at stoichiometrically equivalent doses of 3.8 Gm.
All values are in milligrams per 100 ml. of whole blood.

TABLE 8—Apparent Volume of Distribution of Sulfabromomethazine

Animals	Plasma concentration			Extrapolated zero hour concentration	Dose (mg./kg.)	Apparent volume distribution*
	Time (hours)					
	1	2	4			
1398	43.1	33.2	31.6	56	214	38%
1423	42.8	42.1	38.8	48	214	45%
1420	30.7	21.6	20.4	39	135	35%

*Apparent volume of distribution $= \frac{\text{dose (mg./kg.)}}{\text{zero hour concentration}} \times 10.$

All values are in milligrams per 100 ml. of plasma.

TABLE 9—Concentrations of Sulfabromomethazine in Milk

Time (hours)	Cows					
	1415*		1416*		1420†	
	Blood	Milk	Blood	Milk	Blood	Milk
0	0.0	0.0	0.0	0.0	0.0	0.0
1	0.9	0.6	0.3	0.1	25.3	4.1
2	1.3	0.4	0.7	....	23.2	1.4**
4	2.5	0.8	2.1	0.2	22.2	5.6
6	3.5	0.8	5.2	1.1	19.5	4.8
9	5.6	0.8	6.8	1.1	19.4	4.2
12	6.6	1.0	6.3	1.4	16.4	3.1
24	11.2	1.1	9.8	1.6	9.9	1.9
36	10.4	1.2	6.2	1.7	5.7	1.2
48	9.1	....	5.3	....	1.8	....
60	7.1	1.1	2.2	1.4	0.0	0.5
72	4.0	0.8	1.8	0.7	0.0	0.0

* Given 1.5 gr. per pound (214 mg./kg.) orally; † given 0.94 gr. per pound (134 mg./kg.) intravenously; ** this value was redetermined twice, with the same result—no explanation is readily available for this apparent discrepancy.
All values are in milligrams per 100 ml.

brospinal fluid levels to plasma levels is about 0.1 : 0.2 for the first two days after treatment. After the plasma levels have started to decline, however, the cerebrospinal fluid levels remain at approximately the same values or decline at a rate slower than the plasma values. This lag in disappearance of the drug from the spinal fluid is shown by the increasing ratio of spinal fluid to plasma concentrations.

Urinary Excretion of Sulfabromomethazine.—Two calves were put in metabolism cages and the total urine output of each was collected for a 24-hour period. They had free access to water, and the ration consisted of dairy concentrates and alfalfa hay. The urine for each 24-hour period was collected in a clean bucket, its volume measured, and an aliquot retained for free and total sulfonamide concentration.

By appropriate calculations, the amount of sulfonamide excreted in the "free" and conjugated form for each 24-hour period could be determined. The results are shown (table 11, graph 2). About 75 per cent of the dose was excreted in the six days after administration of the drug; about 20 to 25 per cent of the dose was excreted in the free form, and 50 per cent was excreted in the conjugated form. The degree of acetylation in urine averaged 70 per cent of the total amount excreted, compared to about 12 per cent acetylation in blood. The percentage of acetylation tended to increase at five to six days postadministration, suggesting that the longer the drug remained in the body, the more liable it was to metabolic change.

It also is of interest that the greatest incremental increase in excretion occurred during the first and second day after treatment. This would correspond to the period of most rapid depletion of blood levels. During the latter part of the experiment, when the blood levels were low, the daily increments of sulfonamide excretion became smaller. It is felt that this pattern of urinary excretion of sulfabromomethazine is qualitatively consistent with what would be predicted from the blood level data.

Toxicity of Sulfabromomethazine.—The intravenous administration of doses approximating 1 gr. per pound or greater caused excitement, ataxia, and weakness. If administration was continued, more alarming signs appeared, including dyspnea and convulsions. The possibility existed that these signs were due to the concentration of drug that was used (12.5%) and that lower concentrations would be nontoxic. McManus *et al.*,[11] using intravenous solutions, did not observe toxic effects in cattle, although Fehrenbacher[6] and Bixler[2] observed that 4 of 53 cows treated with a 5 per cent solution showed an acute collapse reaction and that 1 of the 4 died.

It was thought that this acute syndrome might be ascribable to the high alkalinity of the sulfonamide solutions used. However, 3 calves treated with sulfabromomethazine intravenously (214 to 314 mg./kg.) showed ataxia and collapse, although their plasma carbon dioxide combining power did not show a significant change over a 20- to 30-minute period. Conversely, 1 calf given 260 ml. of 5×10^{-3} M sodium hydroxide solution (an amount stoichiometrically equivalent to that contained in the lesser of the sulfonamide doses) did not show signs, in spite of a slight rise in carbon dioxide combining power. This indicates that it was the intrinsic properties of the drug itself, rather than its alkalinity that was responsible for these acutely toxic effects.

It was also found that dogs given single intravenous doses of sulfabromomethazine developed clonic convulsions within one

TABLE 10—Cerebrospinal Fluid Levels in Calves

Time (hours)	Calves†														Plasma* average	CSF* average	Ratio of CSF to plasma
	1319		1329		1342		1346		1347		1349		1350				
	P	CSF	P	CSF	P	CSF	P	CSF	P	CSF	P	CSF	P	CSF			
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	....
12	9.2	2.4	6.1	1.2	7.9	....	7.9	....	9.4	....	9.9	....	11.4	....	7.7	1.8	0.2
24	9.4	....	7.7	....	8.7	....	11.4	....	10.9	....	10.8	....	11.5	1.0	11.5	1.0	0.1
36	7.2	1.1	5.2	0.7	7.9	....	10.0	....	8.2	1.0	8.2	....	9.2	....	6.9	0.9	0.1
60	1.0	1.5	1.9	1.1	3.1	....	4.0	....	3.7	....	1.2	1.8	2.3	....	1.4	1.5	1.1
72	0.5	....	1.0	....	1.5	2.1	1.4	1.6	1.8	0.7	0.6	....	0.6	0.5	1.1	0.9	0.9

* The term "average" as used here refers to an average obtained only from those animals in which concurrent plasma and cerebrospinal fluid levels were obtainable; it is inadvisable to take cerebrospinal fluid samples more often than every 24 hours. † The calves were given sulfabromomethazine orally, at doses of 1.5 gr. per pound (214 mg./kg.).

All values are in milligrams per 100 ml. of fluid. P = plasma; CSF = cerebrospinal fluid.

minute of administration. These were controlled by intravenous pentobarbital sodium.*

In mice, the acute $l.d._{50}$ after intraperitoneal administration was found to be 700 mg. per kilogram of body weight.

Toxic effects such as these were not seen after single oral doses ranging from 214 to 858 mg. per kilogram. Oral doses of 70, 106, and 214 mg. per kilogram, repeated daily for 18 to 21 days, apparently failed to produce inappetence, changes in the hemogram, or gross signs of intoxication. On necropsy, no gross or microscopic lesions were detected that were ascribed to drug administration.† It should be pointed out, however, that some calves developed intercurrent respiratory infections during the course of repeated drug administration. Although these animals were discarded from the experimental trials, it suggests that the responsible organisms were either resistant or not sensitive to the drug.

There was no significant change in the prothrombin time in calves treated with 214 mg. per kilogram of the drug, orally, for three consecutive days.

DISCUSSION

Sulfabromomethazine appeared to have the property of maintaining prolonged blood levels in cattle after oral and intraperitoneal administration of doses of 214 mg. per kilogram (1.5 gr./lb.). The resulting blood levels, although not excessively high, remained above the supposed therapeutic minimum of 5 mg./100 ml. of whole blood for approximately 48 hours after oral administration, and for approximately 40 hours after intraperitoneal administration. The 4 calves that were treated intravenously showed an average duration of blood levels above the generally accepted minimum, lasting for at least 48 hours. However, the variation between individuals indicates the need for further studies before any firm conclusions could be drawn regarding the blood level response to intravenous administration.

The prolonged blood levels, lasting 48 hours after single oral doses of sulfabromomethazine, were substantially more prolonged than those attainable with most other sulfonamides in current use, and of approximately the same duration as those attained with sulfamethazine or sulfamethoxypyridazine.** This latter compound, sulfamethoxypyridazine, has been found to produce prolonged blood levels in cattle,[14] as well as in man,[3,7] after single intravenous or oral doses.

Concerning distribution, the drug seemed to penetrate cerebrospinal fluid and the mammary gland in concentrations that were approximately one fifth of those found in

* In contrast to cattle, dogs did not show a prolonged blood level response to sulfabromomethazine.

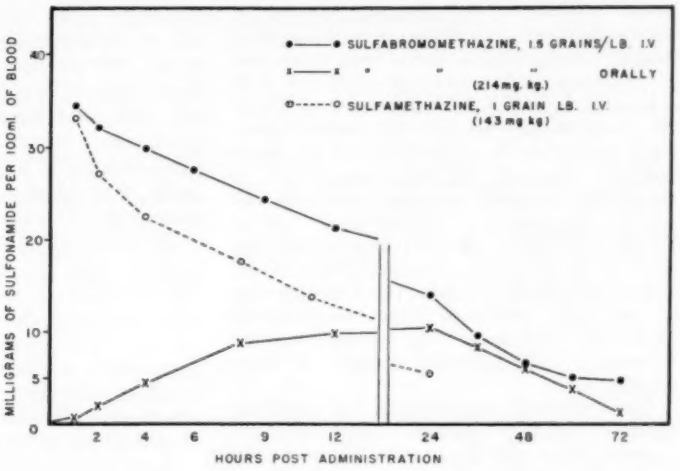
† Some of these calves showed a white mottling of the kidney surface (approx. 3 cm. diameter), a lesion believed due to prior Leptospira infection. Many calves not treated with this or other sulfonamides showed similar lesions.

** 3-sulfanilamido-6-methoxypyridazine (Kynex), Lederle Laboratories, Pearl River, N.Y.

TABLE 11—Urinary Excretion of Sulfabromomethazine by 2 Calves

Interval (hours)	Concentration (mg./100 ml.)		Acetylation (%)	Volume (ml.)	Amount excreted (Gm.)		Dose excreted (%)		Cumulative percentage excreted	
	Free	Total			Free	Total	Free	Total	Free	Total
CALF 1*										
0–24	0.0	0.0	....	1,400	0.00	0.00	....	....	....	....
24–48	50.8	184.0	72	1,030	0.52	1.90	5.4	19.4	5.4	19.4
48–72	93.5	264.0	65	610	0.57	1.61	5.9	16.5	11.3	35.9
72–96	49.5	184.0	73	930	0.46	1.71	4.8	17.6	16.1	53.5
96–120	44.4	109.0	59	600	0.27	0.65	2.7	6.7	18.8	60.2
120–144	15.0	70.0	79	650	0.10	0.46	1.0	4.7	19.8	64.9
144–168	10.2	65.0	85	670	0.07	0.44	0.7	4.5	20.5	69.4
CALF 2†										
0–24	0.0	0.0	....	1,900	0.00	0.00	0.0	0.0	....	....
24–48	61.0	104.0	41	550	0.34	0.57	2.6	4.5	2.6	4.5
48–72	115.0	286.0	60	1,300	1.50	3.72	11.7	29.1	14.3	33.6
72–96	48.5	179.0	73	1,150	0.56	2.06	4.4	16.1	18.7	49.7
96–120	27.5	92.5	70	1,200	0.33	1.11	2.6	8.7	21.3	58.4
120–144	15.0	73.8	80	1,775	0.27	1.31	2.1	10.3	23.4	68.7
144–168	10.0	67.5	85	2,310	0.23	1.56	1.8	12.1	25.2	80.8

* Given an intravenous dose of 1.5 gr. per pound (214 mg./kg.) at 24 hours (9.75 Gm.); † given an intravenous dose of 1.5 gr. per pound (214 mg./kg.) at 24 hours (12.8 Gm.).

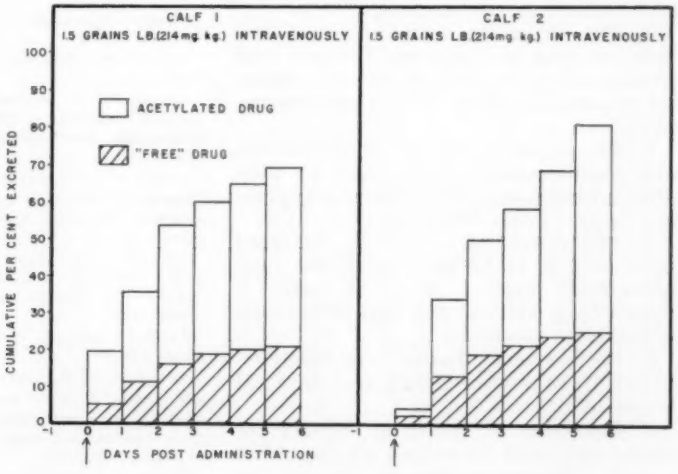


Graph 1—Whole blood levels of sulfabromomethazine in cattle.

blood or plasma. The volume of distribution was found to be in the range of 35 to 45 per cent of body weight. A value such as this suggests a limited degree of cellular penetration. The acetylation in blood was low (12%). This is advantageous, since the acetylated form of sulfonamides is ineffective against bacteria. In the urine, however, acetylation ranged from 41 to 85 per cent of the total sulfonamide present.

Excretion of the drug through the kidney was found to be gradual, and lasted for at least six days after a single intravenous dose of 214 mg. per kilogram of body weight. In 2 calves, 70 to 80 per cent of the dose could be accounted for in both the free and acetylated form. Although the solubility of the drug was found to be low, it is thought that the slow rate of excretion resulted in urine concentrations that were less than those required to reach saturation and cause precipitation. Another possibility is that a portion of the drug is excreted in the form of a more soluble glucuronide or ethereal sulfate.

The acutely toxic effects of sulfabromomethazine appear serious. It was thought initially that the effects seen after intravenous administration were due to drug concentration or the alkalinity of the solu-



Graph 2—Cumulative urinary excretion of sulfabromomethazine in calves.

tions. However, the clinical reports of Fehrenbacher[6] and Bixler[2] indicate that acutely toxic effects were seen with the intravenous administration of 5 per cent solutions. It is felt that intravenous use of the drug should be avoided until further light is shed on the mechanism of the acute collapse reaction. In this connection, it should be pointed out that other sulfonamide solutions occasionally have caused an acutely toxic reaction similar to that described. With respect to oral and intraperitoneal administration, the drug appears to be safe, although some reservations should be held regarding the latter route because of the potential danger of peritonitis.

The clinical experiences of Fehrenbacher,[6] Bixler,[2] Bathke,[1] and Merrill[12] indicate that the drug may be useful in the treatment of such infectious diseases of cattle as pododermatitis, acute mastitis, dirarhea, and respiratory conditions. Further clinical studies are needed before the true worth of the drug can be accurately assessed. Cautious and careful evaluation should be the keynote in such clinical trials.

SUMMARY AND CONCLUSIONS

1) Some of the pharmacological properties of a new sulfonamide, sulfabromomethazine, have been described.

2) This agent produced blood levels that were above the generally accepted therapeutic minimum for periods of approximately 48 and 40 hours after doses of 214 mg. per kilogram of body weight, orally and intraperitoneally, respectively.

3) The drug penetrated cerebrospinal fluid and milk in concentrations that were approximately one fifth of the corresponding plasma and whole blood levels, respectively.

4) The apparent volume of distribution was approximately 40 per cent of body weight, suggesting a limited degree of penetration into intracellular water.

5) The degree of acetylation in blood was found to be 12 per cent of the total sulfonamide present, while in urine it ranged from 41 to 85 per cent of the total sulfonamide present.

6) Sulfabromomethazine is relatively insoluble at the physiologically important pH ranges of 6.0 to 8.0.

7) Excretion of the drug through the kidney continued for at least six days after a single intravenous dose of 214 mg. per kilogram. The concentrations of drug in the urine generally were below the point of *in vitro* precipitation. None of the animals studied, including those given doses two and four times the suggested therapeutic dose, as well as those given repeated oral doses for 18 to 21 days, showed crystalluria or evidence of kidney damage on necropsy.

8) Sulfabromomethazine caused an acutely toxic syndrome characterized by weakness, ataxia, and collapse in some cattle after intravenous administration of both 5.0 and 12.5 per cent solutions. It is suggested that intravenous use of the drug be avoided, for the present at least. Toxic effects such as these were not seen after intraperitoneal or oral administration.

9) Lesions attributable to the drug were not found in calves treated with single intraperitoneal or intravenous doses. Lesions also were absent in calves given repeated oral doses of the drug. Repeated oral doses in a small number of calves did not induce significant changes in the hemogram.

10) Limited clinical trials of the drug in various infectious diseases of cattle suggest that the drug may become useful clinically. In the event that further clinical application is attempted, it is suggested that the dose of sulfabromomethazine be 1.5 gr. per pound of body weight, orally, every 48 hours.

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A Study of the Necrotizing Action of Staphylococcic *Alpha* Toxin

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ONE of the principal causes of gangrenous mastitis in dairy cows is *Micrococcus pyogenes*. Of the toxins produced by some strains of staphylococci, the *alpha* toxin has necrotizing and lethal properties. To understand better the necrotizing action and help explain how these organisms might produce gangrene in the cow's udder, experiments were carried out to determine how the *alpha* toxin causes skin necrosis in rabbits when injected intradermally.

Such a necrosis was first produced by Parker,[14] in 1924, with sterile culture filtrates of staphylococci. This phenomenon was later attributed to the *alpha* toxin.[8] When injected intravenously, the toxin produces necrosis of the renal cortex and also has a lethal effect.[3] Trueta *et al.*[21] and Thal[19] have shown that the *alpha* toxin produces renal necrosis by causing vasoconstriction of the renal arteries. It was considered, therefore, that the vasoconstricting action might be responsible for the necrosis associated with the intradermal injection of rabbits with the toxin.

During the progress of the experiments described in this paper, Thal and Egner[20] demonstrated that the *alpha* toxin causes spasm of the arteries and veins, which results in an ischemic necrosis. The results presented in this paper confirm their findings. Evidence also is presented to show that the mechanism of vasoconstriction probably is not neurogenic.

MATERIALS AND METHODS

Toxin.—The strain of *M. pyogenes* (P-81308) used to produce the *alpha* toxin was isolated originally from a turkey with staphylococcosis. The organism produces only *alpha* toxin, as shown by the fact that the toxin lyses rabbit but not human erythrocytes and is neutralized by *alpha* but not *beta* antitoxin. Toxin was produced by growing the organisms in a semisolid medium consisting of veal infusion broth, 2.0 per cent proteose peptone, 0.5 per cent sodium chloride, and 0.3 per cent agar. Petri dishes, one half full of the medium, were inoculated with 0.5 ml. of 18- to 24-hour broth cultures. The plates then were incubated for 48 hours at 37 C. in an atmosphere which was 20 per cent carbon dioxide and 80 per cent oxygen at the start of incubation. After incubation, the agar was removed by filtration through filter paper, the filtrate centrifuged, and the supernatant fluid sterilized by filtration through a No. 8 or 9 Mandler filter.

Because the studies were carried out sporadically over a period of several years, five batches of toxin were used. The potency of the toxin was estimated by determining the hemolytic titer for rabbit erythrocytes. In addition to testing the undiluted culture filtrate, serial twofold dilutions from 1:25 to 1:25,600 were made with physiological saline solution in tubes measuring 12 by 75 mm., each tube containing 1 ml. of diluted filtrate. One milliliter of a 1 per cent saline suspension of washed rabbit erythrocytes then was added to each tube. The tubes were incubated at 37 C. for 70 minutes, instead of the conventional 60 minutes, to compensate for a slower rise in temperature of the mixtures in an incubator, as compared with a water bath.[4] After incubation, the tubes showing hemolysis but still containing unlysed cells were centrifuged at approximately 1,000 r.p.m. for five to ten minutes, and the amount of hemolysis determined by comparison with a set of standard tubes.[16] After readings were made, all tubes containing unlysed cells were tipped to resuspend them and then placed in the refrigerator for approximately 18 hours. The next day, readings were made again, after centrifugation of those tubes showing partial hemolysis, and the final hemolysin titer was determined from these readings.

The standard set of tubes was prepared by adding 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, and 0.1 ml. of the 1.0 per cent suspension of rabbit erythrocytes to nine tubes (12 by 75 mm.). Each tube was made up to a 2-ml. volume by adding distilled water, which lysed the cells. After the different periods of incubation, each tube that contained toxin and showed partial hemolysis was compared with the standard tubes, and the amount of hemolysis was recorded as the number of the standard tube it more nearly matched. Complete hemolysis was recorded as 10, no hemolysis as 0. The hemolysin titers given in this paper indicate the highest dilution showing 100 per cent hemolysis and that dilution showing 50 per cent hemolysis. If no tube showed 50 per cent hemolysis, the titer was estimated by interpolation.

From the Animal Disease and Parasite Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md.

Unless otherwise stated, the rabbits always were injected with undiluted toxin. No attempt was made to use a standard test dose of toxin because of the loss in potency and the resulting problem of continual restandardization whenever the toxin was used over an extended period. The undiluted toxin always was potent enough to produce necrotic lesions of satisfactory size in susceptible rabbits.

International Standard staphylococcic *alpha* antitoxin* was used to determine the hemolytic test (Lh) doses for three of the five batches of toxin used. The Lh dose of toxin is that amount of toxin which produces 50 per cent hemolysis of the erythrocyte suspension when mixed with 1 unit of antitoxin.

Partial purification of one batch of toxin was carried out at a temperature of −5 C., a pH of 4.0, a final concentration of 15 per cent methanol, and an ionic strength between 0.11 and 0.17, as described by Wittler and Pillemer.[22] The precipitate was resuspended in physiological saline solution and adjusted to a final pH of 7.1 with 1 N sodium hydroxide.

Trypan Blue.—A 1 per cent saline solution of trypan blue was injected intravenously in 10-ml. amounts per rabbit. The solution was sterilized by filtering it through a No. 8 Mandler filter.

Drugs.—A dose of 2 mg. of N-phenoxyisopropyl-N-benzyl-β-chloroethylamine hydrochloride (Dibenzyline†) per kilogram of body weight, based on that used in dogs by Hamilton and Horvath,[11] was given intravenously and produced signs of drowsiness and a flushed or reddened appearance of the skin on the back. One rabbit died shortly after the injection of this dose.

Tetraethylammonium chloride (Etamon**) was given intramuscularly at doses of 10 and 20 mg. per kilogram of body weight. The 20-mg. per kilogram dose is recommended by the manufacturer as the maximum intramuscular dose for man, although this may not have been the best dose to produce the desired vasodilatation in rabbits.

Hair Removal.—The backs of all rabbits were prepared for injection by the removal of hair with clippers and a depilatory containing calcium thioglycollate.

EXPERIMENTAL PROCEDURES AND RESULTS

The development of the skin reaction and the subsequent formation and loss of a scab over a period of several weeks after the intradermal injection of toxin have been well described by Parker [14] and Thal and Egner.[20] The description given by Parker would indicate that blanching occurs one to two hours after injection of the toxin, but not in all rabbits. Thal and Egner stated that, within ten minutes, an area of hyperemia surrounds the site of injection and enlarges progressively over a period of two to four hours, after which the affected area becomes increasingly pale.

In addition, some variations in the appearance of the lesions have been observed by us. During the first five minutes after intradermal injection of the toxin, the surrounding area may become blanched. It becomes progressively larger and grayish, with a peripheral white zone. After about 15 minutes, small isolated areas of a brownish color appear in the gray zone. After one hour, the brown areas are pronounced and give a mottled appearance to the lesion. The pores or porelike structures within this area are dark red. The peripheral white or blanched zone still surrounds the area. Some lesions become grayish purple and are surrounded by a white zone at the end of five minutes; by the end of two hours, they are darker and more purple. In other lesions, a blanched area surrounded by a zone of hyperemia is seen at the end of ten minutes, with the blanched area changing to characteristic gray, surrounded by a blanched zone, after 30 minutes. After several hours, the lesions may be reddened areas, mottled with gray and surrounded by blanched zones.

The differences in appearance of lesions also are evident at the end of 48 hours (fig. 1B). As the lesions become more desiccated, a brownish scab of leather-like consistency is formed. Usually, the lesions do not develop symmetrically but enlarge to a greater extent laterally, as the toxin tends to spread down the side of the rabbit. In this paper, the dimensions given for any lesion indicate the points of maximum length and width.

The immediate and continued blanching produced by the toxin indicated that the dry necrosis might be caused by prolonged local ischemia similar to the cortical necrosis produced in kidneys by prolonged vasoconstriction. To study the effect of the toxin on the blood flow in the affected area, we used the method of injecting trypan blue intravenously as described by Menkin.[13] After it was found that the dye was excluded from lesions 24 hours old, several intradermal injections of toxin were made

* Obtained from the Biological Control Laboratory, National Institutes of Health, Bethesda, Md.

† The Dibenzyline was supplied by the Smith, Kline, and French Laboratories, Philadelphia, Pa.

** Etamon is produced by Parke, Davis and Co., Detroit, Mich.

on the backs of rabbits at different sites and at different times, ranging from 15 minutes up to 24 hours, to determine how soon the dye would be excluded from the lesion after the injection of the toxin. It was found that the dye was excluded from all lesions 30 minutes old and older (table 1). The dye also was excluded from two of three lesions which were 15 to 20 minutes old; however, on close observation, the two unstained lesions appeared mottled because dye was present in the porelike structures mentioned previously. The other lesion stained more intensely than the surrounding normal skin, similar to areas of inflammation produced by the intradermal injection of 0.1 ml. of xylene. When 0.2-ml. doses of xylene were injected intradermally, large intensely stained areas developed around small unstained zones which eventually became necrotic. The intradermal injections of sterile filtrates of control mediums, which contained no toxin, produced evidence of inflammation in some rabbits and no effect in other rabbits. The unstained areas affected by toxin were usually surrounded by a narrow zone of intense staining, similar to that seen in areas of inflammation.

Burky [6] reported that rabbits less than 4 months old did not react to the necrotizing or lethal action of staphylococcic toxin. The rabbits were considered nonreactive, either because they were immune or because they were not equipped with a mechanism to respond to the action of the toxin. This observation was investigated further by injecting the toxin intradermally in 17- to 58-day-old rabbits. The results (table 2) are contradictory to those reported by Burky, and indicate that rabbits under 2 months of age can be susceptible to the necrotizing action of *alpha* toxin.

Because it has been suggested that *alpha* toxin is a neurotoxin,[5] it was considered possible that the vasoconstricting or lethal action of the toxin was produced by its action on vasoconstrictor nerve fibers. In the case of Shigella toxin, it has been reported by Penner and Klein [15] that the mucosal congestion, edema, and superficial erosions of the duodenum and jejunum, and the edema and hemorrhage in the gallbladder produced by this toxin did not result by direct action of the toxin on the tissues, but indirectly by action on the central nervous system. The lesions were considered produced by a vascular mechanism which is under control of the sympathetic nervous system. Paralysis of the latter at the myoneural junction or in the ganglia by means of drugs prevented the formation of lesions. Therefore, attempts were made to counteract any possible neurotoxic action of the *alpha* toxin by the sectioning of the sciatic nerve and by the use of procaine, Dibenzyline, tetraethylammonium chloride, and histamine (table 3).

According to Best and Taylor,[2] the vascular nerves to the distal part of the limbs are carried to the peripheral vessels via the somatic nerve trunks, which, in the

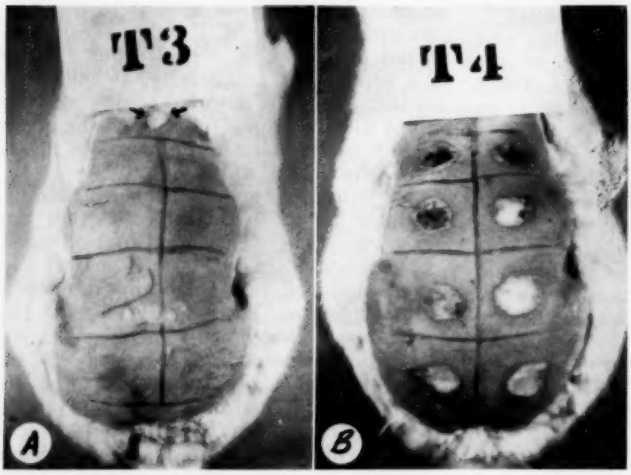

Fig. 1—A comparison of the susceptibility of 2 rabbits to the dermonecrotizing action of varying dilutions of alpha toxin. The arrows on rabbit T3 indicate a lesion produced by the injection of undiluted toxin. The lesions are 48 hours old, and trypan blue was injected intravenously before the pictures were taken.

TABLE 1—The Relationship of Time to the Local Effect of Alpha Toxin as Shown by the Exclusion of Trypan Blue from the Area Infiltrated with Toxin

Rabbit No.	Age of lesion at time of intravenous injection of trypan blue: 2 to 3 hours	1 to 2 hours	45 to 50 minutes	30 to 35 minutes	15 to 20 minutes	Substance and amount injected intradermally		Toxin titer: 100% hemolysis	50% hemolysis
206	.....	Neg.	Neg.	Neg.	.....	Toxin	0.2 ml./site	1:1,480	1:10,600
	.....	.....	Pos.	.....	.....	Control medium	0.2 ml./site		
219	.....	Neg.	Neg.	Neg.	.....	Toxin	0.2 ml./site	1:1,480	1:10,600
	.....	.....	Pos.*	.....	.....	Xylene	0.2 ml./site		
693	.....	Neg.	.....	Neg.	Pos.	Toxin	0.2 ml./site	1:1,200	1:8,900
	.....	.....	.....	Pos.	.....	Xylene	0.1 ml./site		
207	Neg.	Neg.	Neg.	Neg.	Neg.*	Toxin	0.1 ml./site	1:360	1:4,300
209	Neg.	Neg.	Neg.	Neg.	Neg.*	Toxin	0.1 ml./site	1:360	1:4,300
	.....	.....	.....	.....	Pos.	Xylene	0.1 ml./site		

Neg. = complete exclusion of dye from the affected area when observed 20 to 30 minutes after the intravenous injection of the dye; Neg.* = partial exclusion of dye as described in the text; Pos. = intense staining of the affected area as compared with the surrounding normal skin, which was stained a lighter blue; Pos.* = intense staining of the affected area, with a small centrally located unstained zone.

case of the hindlegs of an animal, is the sciatic nerve. It is through the sympathetic fibers traveling with somatic nerve trunks that constrictor impulses are conveyed to the blood vessels. Since section of a peripheral nerve probably would cause complete degeneration of vasoconstrictor fibers and might prevent the development of necrosis, a neurectomy was performed on 2 rabbits, on the left sciatic nerve close to its emergence through the greater sciatic foramen. Thirty days after the legs became paralyzed, the normal and the neurectomized legs in each rabbit were injected intradermally with toxin, at two points in the region between the head of the femur and the point of the hock. Necrotic lesions developed in both legs. The lesions were slightly smaller in the paralyzed legs, probably because the paralyzed legs were atrophied and had a reduced blood supply.

To prevent any possibility that the vasoconstriction resulted indirectly by reflex action following stimulation of sensory receptors, procaine was injected subcutaneously and intradermally 15 minutes before the injection of the toxin at the same site. Necrosis was not prevented when the dosages of procaine shown (table 3) were used locally. Likewise, death was not prevented in 3 of 4 rabbits when the total intravenous dose of procaine (table 3) was divided and given as three equal doses at one-hour intervals and the toxin was administered intravenously 15 minutes after the first dose of procaine. The rabbit lived, probably because it was immune rather than because it was protected by the procaine.

Dibenzyline, a chemical derivative of N-(2-chloroethyl)-dibenzylamine hydrochloride (Dibenamine*), belongs to a group of adrenergic blocking agents which act at the neuroeffector junctions to block direct sympathetic stimulation by epinephrine.[1,9] Therefore, it was used with the idea that the necrotizing action of the *alpha* toxin could be blocked if the toxin caused vasoconstriction in a manner similar to epinephrine.

The toxin was injected intradermally in each of 3 rabbits one hour before, simultaneously with, and 30 and 60 minutes after the intravenous injection of the drug. In a fourth rabbit, the drug was injected intradermally with the toxin, at the same site. Two other rabbits were given the drug 40 minutes before intravenous injection of the toxin to determine if the lethal action of the toxin could be prevented. The fact that necrotic lesions did not develop in 1 of the rabbits (table 3) also is attributed to an immunity to the toxin rather than to a protection from the

TABLE 2—The Reactions of Young Rabbits to the Intradermal Injection of 0.1 ml. of Undiluted Alpha Toxin*

Rabbit No.	Litter	Age (days)	Weight (Gm.)	Size of necrotic lesion at the end of 48 hours
YR-1	A	17	397	11 x 11 mm.
212	B	17	340	31 x 15 mm.
214	C	17	170	26 x 18 mm.
YR-2	A	23	624	35 x 16 mm.
221	C	30	567	25 x 20 mm.
223	C	30	425	37 x 15 mm.
5526	C	45	865	30 x 21 mm.
5527	C	45	907	62 x 25 mm.
5528	C	58	1,361	26 x 18 mm.
5529	C	58	1,588	26 x 18 mm.

* The hemolysin titers of the toxin ranged from 1:160 to 1:240 for 100 per cent hemolysis and 1:1,500 to 1:2,700 for 50 per cent hemolysis during the test period.

* Dibenamine is produced by Smith, Kline, and French Laboratories, Philadelphia, Pa.

drug. No protection from the lethal effects of the toxin was obtained with the dosage of drug used. The variability in the susceptibility of rabbits to the action of *alpha* toxin will be discussed.

Tetraethylammonium chloride produces vasodilatation by its paralytic action upon sympathetic ganglia, thereby blocking tonic vasoconstrictor impulses.[2] If the toxin acted indirectly through the central nervous system, any vasoconstrictor impulses would probably be blocked by this drug or, if the toxin acted directly on the blood vessels, its vasoconstricting action might be counteracted by the vasodilatation produced by the drug. Therefore, the same schedule of inoculation of toxin as described for Dibenzyline was used in 6 rabbits treated with tetraethylammonium chloride, except that the drug was given intramuscularly for its systemic effect. The dosages used gave no protection against the necrotizing or lethal action of the toxin.

Histamine was injected intradermally and subcutaneously and, 15 minutes later, the toxin was injected at the same site. The hyperemia which results from a dilatation of the arterioles, venules, and capillaries [17] was considered another possible means of counteracting the action of the toxin. As used, the drug failed to prevent necrosis.

The hemolysin titers of the toxins used to challenge all rabbits (table 3), except the 2 neurectomized rabbits and the 4 treated locally with procaine, were 1:200 for 100 per cent hemolysis and 1:2,400 for 50 per cent hemolysis. The other 6 rabbits were given toxin with minimum titers of 1:1,200 for 100 per cent hemolysis and 1:8,900 for 50 per cent hemolysis.

During these studies, some rabbits showed differences in their susceptibilities to either the necrotizing or the lethal action of the *alpha* toxin. As illustrated (fig. 1), the backs of the rabbits T3 and T4 were divided into eight areas, with the transverse line at the shoulders acting as the anterior border and the line between the tuber coxae as the posterior border. Both rabbits were injected intradermally with 0.1-ml. amounts of 1:10, 1:20, 1:30, and 1:40 dilutions of toxin in saline solution. On the left side of each rabbit, the various dilutions were injected in successive order, so that the 1:10 dilution was injected in the region next to the shoulder, and the 1:40 dilution in the region next to the tuber coxae. On the right side of each rabbit, the order of injection was reversed. When no immediate blanching developed in rabbit T3 after injection of the various dilutions of toxin, 0.1 ml. of undiluted toxin was injected anterior to the transverse line at the shoulders. The resulting lesion was a blanched area (fig. 1A), showing that the rabbit was not completely resistant to the toxin.

The presence of scabs in the ear canals of rabbit T3 was indicative of ear mange, although no attempt was made to demonstrate the presence of mites. In 2 other rabbits which were resistant to the toxin, there was evidence of ear mange. The scabs were removed from an ear of 1 of these, and the raw surfaces swabbed and then cultured on blood agar. Coagulase-positive, α-, β-hemolytic staphylococci were isolated readily from the plates after incubation. Infection with these organisms may have stimulated the production of *alpha* antitoxin and resulted in the increased resistance of this rabbit.

These findings indicate that one factor contributing to the variation in susceptibility to the action of *alpha* toxin may be ear mange, with a concomitant staphylococcic infection.

It had been observed that the skin in the posterior part of the backs of some rabbits apparently is less sensitive to the necrotizing action of the *alpha* toxin than the skin in the anterior part of the back However, in using this pattern of injection (fig. 1), the results in 7 of 10 rabbits which developed lesions were inconclusive. The same dose of toxin produced larger lesions in the anterior part of the back in 7 of 14 paired injections. The posterior lesions were larger in 4 rabbits and, in the other 3, the anterior and posterior lesions were equal in size.

Two Holstein-Friesian cows were injected intradermally with toxin to determine if necrosis would develop in a manner similar to that observed in rabbits. Injections of toxin were made in unpigmented, shaved areas of skin. Lesions developed in a manner similar to those in rabbits, except that the lesion had a purplish discoloration until a scab formed. In 1 cow, the injections were made in the left neck region and, in the other cow, in the dorsal region of the right shoulder. Areas

of induration which developed around the lesions in the neck region by the end of 48 hours were still evident at the end of six days. It is possible that the indurations were not evident in the shoulder region because the skin was tighter and the lesions could not be readily palpated. The scabs in both cows appeared more superficial than those seen in rabbits, which may be attributed to the thicker skin of the cow. The skin reactions in the cows were similar to those produced in guinea pigs, which also have a thicker skin and are less sensitive to the action of the toxin than rabbits.[3]

The production of skin necrosis in the cow would indicate that the toxin probably acts in a similar manner in producing gangrenous mastitis. Whether the toxin produces gangrene in the udder by its vasoconstricting action has not been shown; however, in the cow with gangrenous mastitis (fig. 2), there is clinical evidence that it does. From external appearances, it would seem that the left, rather than the right, quarter had been infected. Actually, the gangrenous mastitis developed in the right rear quarter and extended over the posterior and lateral surfaces of the left rear quarter. The milk from the left rear quarter was off-color for several days, due to the presence of blood, but at no time were any hemolytic, coagulase-positive staphylococci isolated from this quarter. However, three separate isolations of pathogenic staphylococci were made from the right rear quarter during the first 19 days. Considering the complex blood circulatory system of the udder with its anastomosing vessels,[18] the gangrene in the left rear quarter might have resulted from a constriction of the vessels in the posterior medial part of the right rear quarter.

Sometimes, in fulminating and gangrenous mastitis, there are signs which appear to be neurogenic. The cows may show partial paralysis of both hindlegs, with flexion of the fetlocks while standing, marked incoordination when forced to walk, inability to rise, little or no reaction when the tail is manipulated, and no response from pricking the skin with a needle posterior to the tuber coxae.

To determine if the *alpha* toxin produces these signs by its action on nerves, we injected toxin epidurally between the first and second coccygeal vertebrae in a 7-month-old bull calf and a 2-year-old heifer. The toxin had been partially purified by alcohol precipitation, and the hemolysin titers were 1:1,600 for 100 per

TABLE 3—Methods Used to Counteract the Necrotizing and Lethal Actions of Alpha Toxin

Treatment	Drug		Toxin		Results	No. rabbits
	Method of injection	Dosage	Method of injection	Dosage		
Neurectomy (sciatic nerve)	....	....	i.d.	0.1 ml.	Skin necrosis	2
Procaine HCl (2% soln.)	s.c.	0.5 ml.	i.d.*	0.2 ml.	Skin necrosis	2
	s.c.	1.0 ml.	i.d.*	0.2 ml.	Skin necrosis	1
	i.d.	0.5 ml.	i.d.*	0.2 ml.	Skin necrosis	1
	i.v.	0.4 ml./kg.	i.v.	0.5 ml./kg.	Died	1
	i.v.	1.3 ml./kg.	i.v.	0.5 ml./kg.	Died	2
	i.v.	1.3 ml./kg.	i.v.	0.5 ml./kg.	Lived	1
	i.v.	1.3 ml./kg.	....	....	No ill effects	1
Dibenzyline	i.v.	2.0 mg./kg.	i.d.	0.2 ml.	Skin necrosis	1
	i.v.	2.0 mg./kg.	i.d.	0.1 ml.	Skin necrosis	1
	i.v.	2.0 mg./kg.	i.d.	0.1 ml.	No skin necrosis	1
	i.d.	0.5 mg.	i.d.*	0.1 ml.	Skin necrosis	1
	i.v.	2.0 mg./kg.	i.v.	0.5 ml./kg.	Died	2
Tetraethyl ammonium chloride	i.m.	10.0 mg./kg.	i.d.	0.2 ml.	Skin necrosis	1
	i.m.	20.0 mg./kg.	i.d.	0.1 ml.	Skin necrosis	2
	i.d.	10.0 mg.	i.d.*	0.1 ml.	Skin necrosis	1
	i.m.	20.0 mg./kg.	i.v.	0.5 ml./kg.	Died	2
Histamine HCl (1:500 dil.)	i.d.	0.1 ml.	i.d.*	0.1 ml.	Skin necrosis	1
(1:500 dil.)	s.c.	0.2 ml.	i.d.*	0.1 ml.	Skin necrosis	
(1:1000 dil.)	i.d.	0.1 ml.	i.d.*	0.1 ml.	Skin necrosis	1
(1:1000 dil.)	s.c.	0.2 ml.	i.d.*	0.1 ml.	Skin necrosis	

* Toxin was injected at the same site in which the drug was injected.
i.d. = intradermal; i.m. = intramuscular; i.v. = intravenous; s.c. = subcutaneous.

cent hemolysis and 1:17,000 for 50 per cent hemolysis. The Lh dose was 0.095 ml., and 0.1 ml. of a 1:80 dilution of the toxin produced necrosis when injected intradermally in a rabbit. Dilutions higher than 1:80 were not tested. About 40 minutes after 10 ml. of the toxin had been injected epidurally in the calf, it started straining to defecate. For a 20-minute period before this, the calf was restless and lay down and got up several times. Intermittent straining continued, with little passage of feces. During a 10-minute period, 50 to 60 minutes after the injection, the calf strained six or seven times per minute. However, one hour and 45 minutes after the injection, it was chewing its cud and not straining. At no time was there any evidence of paralysis.

Fig. 2—Gangrenous mastitis in the right rear quarter of a cow's udder 19 days after the onset of signs, with necrosis extending over the non-infected left rear quarter.

In the heifer, 20 ml. of toxin was injected epidurally in 10-ml. amounts, with a ten-minute interval between injections. For an hour after being released from the stanchion, the heifer repeatedly attempted to lick the site of injection, appeared restless, and lay down and got up nine times. Two hours later, she appeared normal, and at no time was there any evidence of paralysis.

The inability to produce a paralysis by this method might be attributed to the use of an inadequate amount of toxin, to the fact that the site of injection was inappropriate for demonstrating the neurotoxic action of the toxin, or to the fact that both animals were immune and the toxin was neutralized by antitoxin. The blood *alpha* antitoxin titers were not determined in either animal, although, 11 months earlier, the antitoxin titer of the heifer was negative and there was no history of staphylococcic mastitis during the intervening period. Previous experience with calves and heifers which had no history of staphylococcic udder infection would indicate that the antitoxin titers would be low or absent.[4]

DISCUSSION

The data presented confirm the findings of Thal and Egner[20] that dermonecrosis produced in rabbits with staphylococcic *alpha* toxin is probably the result of an ischemia produced by a prolonged vasospasm. The intravenous injection of trypan blue as a means of studying the local blood flow in the skin showed that the dye was partially excluded from some of the lesions as early as 15 minutes after the toxin was injected intradermally. Vasoconstriction was apparently complete by the end of 30 minutes, as no dye was found in any of these lesions or those that were older.

It was also found that dermonecrosis could not be inhibited by (1) a neurectomy of the sciatic nerve for the purpose of destroying the vascular nerves to the skin of the lower part of the hindleg; (2) the use of the local anesthetic, procaine, or Dibenzyline and tetraethylammonium chloride, which block the vasoconstrictor impulses at the neuroeffector junctions and the ganglia, respectively; and (3) the use of histamine to produce vasodilatation and counteract the vasoconstricting action of the toxin. These negative results suggest that the action of the toxin on the blood

vessels is not neurogenic. It is possible that the toxin acts directly on the blood vessels to produce vasoconstriction in a manner similar to nicotine.[10]

The first evidence of gangrenous mastitis is the appearance of a demarcated blue area of skin, which often involves the teat and is cold to the touch. The fact that dermonecrotic lesions were produced in cows, which were similar to those in rabbits, is indicative that the action of the toxin is the same in both species. Consequently, it is probable that the toxin also produces a prolonged vasoconstriction of the vessels in the udder which results in an ischemia and a stagnant type of cyanosis. In some cases of gangrenous mastitis, stumps of thrombosed vessels can be seen after the sloughing of the necrotic tissue. It might be considered, therefore, that thrombosis is a primary cause of the gangrene. However, it was shown by Thal and Egner[20] that heparinization of rabbits to prevent clotting had no effect on the development of dermonecrosis, which would indicate that the thrombi formed in the udder are probably secondary to the development of the ischemia produced by the vasoconstricting action of the toxin.

It has been suggested that the toxic substance in staphylococcic culture filtrates is a neurotoxin.[5] Paralysis of the hindlegs, which precedes death in some animals,[7] is also seen in some cases of fulminating and gangrenous mastitis. Therefore, *alpha* toxin was injected epidurally in the sacrococcygeal region of 2 young cattle, with the idea that if paralysis was produced it could be assumed that the *alpha* toxin is a neurotoxin. The negative results do not necessarily indicate that the toxin does not act on nerves to produce paralysis, as the dose may have been inadequate, the animals immune, or the site of injection inappropriate for demonstrating neurotoxic properties of the toxin. It does lead to the idea, however, that a vasospasm of the blood vessels of the hindlegs may cause an ischemic type of paralysis. This would be similar to the condition in man in which pressure on the blood vessels of a limb results in it "going to sleep." More work is necessary to determine if this hypothesis is valid.

Some of the rabbits used in these studies were less susceptible to the effects of the toxin than others. In 3 of the resistant rabbits, ear mange was present and hemolytic, coagulase-positive staphylococci were isolated from one of them. The development of an active immunity against the *alpha* toxin could account for the increased resistance. The results showing that rabbits less than 2 months old were susceptible to the necrotizing action of the toxin were contradictory to the idea that young rabbits are less susceptible to the toxin than older rabbits.[6,12] A passive immunity obtained from immune does might explain the greater resistance reported for young rabbits.

Summary

To understand better the development of gangrene of the udder as a result of infection with *Micrococcus pyogenes,* we carried out experiments to determine the way in which dermonecrosis is produced in rabbits after the intradermal injection of staphylococcic *alpha* toxin.

The results indicate that the necrosis is ischemic in nature and is probably caused by the vasoconstricting action of the toxin. The necrotizing action of the toxin could not be inhibited by the sectioning of the sciatic nerve or by the use of procaine, N-phenoxyisopropyl–N–benzyl–β–chloroethylamine hydrochloride (Dibenzyline), tetraethylammonium chloride (Etamon), or histamine. These findings were interpreted as indicating that the toxin probably acts directly on the blood vessels to produce vasoconstriction, rather than indirectly by stimulation of vasoconstrictor nerve fibers.

The failure to produce paralysis in a 7-month-old calf and a 2-year-old heifer by the epidural injection of toxin was considered evidence that the paralysis seen in some cases of acute staphylococcic mastitis may not be neurogenic but, rather, the result of an ischemia caused by the vasoconstricting action of the toxin on the blood vessels of the hindlegs.

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The Effects of Feeding Fluorine, as Sodium Fluoride, to Dairy Cattle—A Six-Year Study

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THE NEED FOR more specific information on the effects produced in livestock by the prolonged ingestion of fluorine* is reflected by the numerous recent studies on this subject.[7, 12, 13, 15, 20]

During the past decade, there has been renewed interest in the problem of chronic fluorine intoxication in cattle. This has come about because of frequent reports of damage or suspected damage to livestock consuming forage in the vicinity of industrial plants known to emit fluorine compounds into the atmosphere. Although chronic fluorosis in cattle has been studied previously, the experiments have been concerned for the most part with the relatively insoluble fluorides found in rock phosphate.[4, 14, 17]

A critical review of the literature[18] indicated that the signs of fluorosis in cattle are mottled, stained, and worn incisors; bilateral lameness; and exostoses of the long bones, jawbone, and ribs. The degree of such changes is dependent upon the chemical form and concentration of the ingested fluorine, the duration of exposure, the age of the animal, physiological stress, and the effectiveness of defense mechanisms.[11]

The literature review failed to reveal, from studies with laboratory animals as well as from feeding experiments with cattle, sheep, and swine, any definitive information on the effect of feeding soluble fluoride-fluorine. Unfortunately, toxicity relationships between soluble and less soluble forms had been based on data from experiments with small animals, with the resulting uncertainties of translating results into values applicable to ruminants. The literature did suggest, however, that a safe level for the ingestion of soluble fluorine would be 1 mg. per kilogram of body weight per day. A field investigation[16] in an area where cattle obtained most of their fluorine through drinking water high in fluorides confirmed this conclusion.

Since there was a lack of information from long-term experiments on the feeding of soluble fluorides to large animals, an experiment with dairy cattle being fed fluorine as sodium fluoride was started in December, 1949. The objective of this experiment was to determine the effects of prolonged feeding of fluorine, beginning with 2-year-old heifers, under conditions of practical dairy management and practice.

EXPERIMENTAL PROCEDURE

Selection of the animals, feeding practices, and milking procedures have been described previously.[19] The feedstuffs were analyzed at intervals throughout the experiment and were found to contain fluorine, on a dry basis, in the following amounts: pasture grass, 3 to 12 parts per million (p.p.m.); grain mixture, 4 to 27 p.p.m.; silage, 1 to 5 p.p.m.; beet pulp, 1 to 5 p.p.m.; and alfalfa hay, 1 to 11 p.p.m. Well water consistently contained a low fluorine level (0.1 to 0.2 p.p.m.). Throughout the experiment, each cow received, on an average, 5 to 8 lb. of grain per day. The commercial grain mixtures were based on barley as the principal ingredient and contained 14 to 17 per cent of crude protein.

With a knowledge of the hay, beet pulp, silage, and grain mixture intake, and from estimations of water[9] and green pasture consumption,[10] calculations indicated that each cow consumed approximately 0.15 to 0.3 mg. of natural fluorine

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* The element fluorine never occurs in the free state in nature, but rather in combination with other elements. Compounds containing fluorine are commonly called fluorides. In this paper, the word "fluorine" refers to the fluorine content of the particular fluoride in question.

per kilogram of body weight per day. Each experimental cow received, while in her stanchion at the time of the morning milking, a calculated amount of fluorine as sodium fluoride (reagent grade) added dry to slightly moistened beet pulp. The total amount of fluorine the animals received daily is given (table 1).

TABLE 1—Total Amounts of Fluorine Given Daily to Cattle

Group (No.)	Cow (No.)	Fluorine (mg./kg. of body weight)
I (control)	1– 4	0.15–0.3*
II	5– 8	1.0
III	9–12	1.5
IV	13–16	2.0
V	17–20	2.5

* Natural fluorine.

Urine samples were collected, at three- to four-month intervals, from single voidations, before the morning feeding of the sodium fluoride supplement. To reduce the variation in individual results, the specific gravity of each urine sample was obtained at 60 F., and the analytical result adjusted to a specific gravity of 1.040. On several occasions, multiple urine samples were taken to study diurnal variations. Blood samples were taken from each cow at the beginning of the experiment and at irregular intervals thereafter. Measurements were made of hemoglobin, hematocrit, and erythrocyte and leukocyte counts, followed by calculations of mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular concentration.

As the experiment progressed, it became apparent that its value would be increased if the fluorine intake were expressed in parts per million (p.p.m.) of the total ration, as well as in milligrams per kilogram (mg./kg.) of body weight. During February and March of the fifth year, each animal still being used in the experiment was put in an individual drylot pen and fed her normal ration for a three-day conditioning period. This was followed by a three-day collection period, during which quantitative measurements were made of grain, beet pulp, hay, silage, and water consumption, and of fecal excretion. Daily aliquots of these materials were analyzed for moisture and fluorine. The milking routine and the feeding of supplemental fluorine were continued as usual.

A physical examination was given each cow monthly. The incisor teeth were examined in detail once a month during the first year and at three- to six-month intervals thereafter. In order to minimize disturbances in lactation performances, detailed examinations of the molar teeth were not attempted.

At the beginning of the experiment, at subsequent six-month intervals, and at slaughter, colored motion pictures and black-and-white photographs were made of each cow. Detailed 35-mm. color photographs of each cow's incisor teeth were taken at the same time.

Statements have appeared in the literature that fluorine causes increased elongation of cattle hoofs. During the fifth year of the experiment, the hoof growth of each animal was measured over a six-month period. A notch was filed 1 inch from the proximal edge of the periople on the lateral surface of the left hind hoof of each animal. Six months later, the distance of this notch from the periople was again determined.

Necropsies of the animals were carried out at a commercial meat-packing house in the presence of a U.S.D.A. meat inspector. The metacarpal, metatarsal, and mandible bones, twelfth and thirteenth ribs, and rib cartilage were removed from each cow for fluorine analysis. Soft tissues saved for fluorine analysis were lung, heart, liver, kidney, and adrenal. At necropsy, samples of these tissues, plus sections of pituitary, thyroid, aorta, peribronchial lymph node, and omentum fat were preserved in formalin for subsequent histological examination. Microscopic examination of the teeth was not attempted.

Bone and fecal samples were analyzed for fluorine by the method of Willard and Winter,[21] as modified by the Aluminum Company of America.[1] Fluorine in soft tissues and urine was determined by the method of the Association of Official Agricultural Chemists.[2] A slight modification of the latter procedure was used in this laboratory: 500 cc., instead of 250 cc., was collected in the distillation step, and then a 200-cc. aliquot was taken for direct titration.

Six months after the experiment was begun, cow 15 (1.5 mg. F/kg. body weight) fell into a shallow ditch during the night and died as a consequence. Cow 6 (1.0 mg. F/kg. body weight) was killed two years and four months after the experiment started, following several months of poor health. Necropsy revealed multiple abscesses of the spleen caused by foreign body penetration.

The experiment was designed to distinguish between damaging and nondamaging boundary levels of fluorine fed in the soluble form as sodium fluoride. It was devised to test the influence of various levels of soluble fluorides on dairy cows over long periods, and particularly its effect on lactation, weight, teeth, general health, and bones and tissues.

RESULTS AND DISCUSSION

Milk Production.—The feeding of fluorine as sodium fluoride to dairy cows, at levels as high as 2.5 mg. of fluorine per kilogram of body weight, for five lactations had no adverse effect on milk production. Average 305-day and total milk production records of each group are presented (table 2). Individual lactation records are not presented here but were tabulated during the experiment. Individual records greater than 150 but less than 305 days were computed as "length-corrected" milk

and butterfat production.[5] "Mature equivalent" production records were calculated for each cow's lactation, based on age-conversion factors developed by the U.S. Department of Agriculture.[8] These data were also calculated in terms of "4 per cent fat-corrected milk."

The individual records were subjected to statistical analysis by the "t" test and analysis of variance. The "t" test showed no statistical differences, in any of the production data, between the control group and groups given 1.5, 2.0, and 2.5 mg. of fluorine per kilogram of body weight per day. The production records of the group fed 1 mg. of fluorine were significantly different, at the 5 per cent level of probability, from those of the controls and of the groups which received higher levels of fluorine. The uniformly poorer production of the cows fed 1 mg. of fluorine was not due to the effect of fluorine *per se*, since cows fed higher levels of fluorine produced as well as or better than the controls. In addition, the traumatic gastritis of cow 6, the "hard" milking characteristics of cow 7, and the failure of cow 8 to carry any calf for a full gestation period adversely affected the production records of this group.

Treatment of the data by analysis of variance showed no differences in milk production, but the pounds of butterfat produced by the fluorine-supplemented groups were significantly less than those produced by the controls. This difference, however, was a reflection of the control cows' abnormal behavior. It is generally accepted that there is no consistent change in fat percentages from one lactation to another. A cow with a low fat test as a heifer will not better it in succeeding years;[9] yet, the controls in this experiment had an average increase in butterfat test for successive lactations. With increases in the percentage of fat in the milk of the controls, the pounds of fat produced seemed to become more disproportionate in relation to the relatively constant percentages of fat in the milk of the experi-

TABLE 2—Average Milk Production Records of Cows Fed Sodium Fluoride, Arranged by Sequential Lactations for Each Level of Treatment

Group	Lactation (No.)	Total daily fluorine (mg./kg. body wt.)	305-day production			Total production		
			Milk (lb.)	Fat (lb.)	Fat (%)	Days in milk	Milk (lb.)	Fat (lb.)
I	1	0.15–0.3	10,441	404	3.85	376	11,925	476
	2		10,125	415	4.07	302	11,116	458
	3		10,789	442	4.02	339	11,773	483
	4		14,502	605	4.13	338	15,949	666
	5		15,956	638	4.00	260†	14,988	593
	6		.....	.....	.....	91†	6,987	277
II	1	1.0	11,109	391	3.53	319	11,264	423
	2		9,617	356	3.71	289	9,634	357
	3		10,580	382	3.62	269	8,664	318
	4		11,897	430	3.67	313	12,006	444
	5		12,353	434	3.50	351	13,091	462
	6		.....	.....	.....	153†	6,327	228
III	1	1.5	10,636	403	3.82	307	10,929	415
	2		11,266	425	3.81	368	12,469	476
	3		12,323	488	3.85	312	12,422	480
	4		17,677	652	3.79	314	15,997	612
	5		15,640	581	3.72	307	15,841	590
	6		.....	.....	.....	155†	11,008	422
IV	1	2.0	10,677	380	3.55	277	10,828	386
	2		12,559	474	3.87	344	13,299	507
	3		13,212	403	3.57	321	13,433	480
	4		17,265	625	3.63	349	18,498	672
	5		16,731	610	3.65	353	17,839	654
	6		.....	.....	.....	143†	8,767	297
V	1	2.5	11,440	378	3.31	322	11,842	389
	2		10,716	385	3.65	330	11,432	410
	3		13,273	455	3.41	327	14,283	490
	4		14,488	530	3.59	331	15,595	557
	5		16,022	536	3.35	271†	13,772	470
	6		.....	.....	.....	116†	7,821	253

* Based on a naturally completed lactation period of at least 150 days; † incomplete lactations—cows removed from experiment for slaughter data.

mental groups. The individual fat percentages suggest that there was an inherent capacity of cows in the control group to produce 4 per cent or more butterfat, compared with a butterfat production of 3.4 to 3.8 per cent for almost all the other cows. More important, however, no cow given supplemental fluorine developed a downward trend in butterfat percentages with successive lactations.

Individual lifetime milk and butterfat production and the average daily production of milk and butterfat, based on days in milk and days in the herd, are presented (table 3). The total number of cow-days in the herd was the same for the control group and for groups fed 1.5 and 2.5 mg. of fluorine. Yet the lifetime milk production of the fluorine-supplemented groups exceeded that of the controls by 35,000 lb. in each case.

These data also show that the duration of lactation was not adversely affected by fluorine. The "total days in the herd" were the same; the control group was "in milk" 79 per cent of the time, whereas the "in milk" time factor for the fluorine-supplemented groups ranged from 83 to 86 per cent.

The "t" test was applied to the lifetime fat production of all groups. Comparisons were made using all animals in each group and again after elimination of the poorest fat producer in each group. In no case was there a significant difference between the control and fluorine-supplemented groups in lifetime fat production. Furthermore, conversion of lifetime records to an average daily production basis in terms of "days in milk" and of "days in the herd" re-emphasized that fluorine, as sodium fluoride, in the range of 1.0 to 2.5 mg. per kilogram of body weight had no significant effect on milk or butterfat production for adult dairy cows maintained under conditions of practical farm management.

Reproduction.—At the start of the experiment, the newly purchased heifers were already pregnant by natural breeding. Subsequent breedings were by arti-

TABLE 3—Lifetime Milk Production of Cows Fed Sodium Fluoride

Cow (No.)	Total daily fluorine (mg./kg. body wt.)	Lifetime production		Total days in milk	Total days in herd	Average daily production			
						Based on days in milk		Based on days in herd	
		Milk (lb.)	Fat (lb.)			Milk (lb.)	Fat (lb.)	Milk (lb.)	Fat (lb.)
1	0.15–0.3	90,911	3,673	1,904	2,146	47.8	1.93	42.4	1.71
2		26,582	978	1,064	1,471	25.0	0.92	18.1	0.66
3		46,932	2,050	1,292	1,906	36.3	1.58	24.6	1.08
4		75,586	3,089	1,766	2,089	42.8	1.75	36.2	1.48
Group total		240,011	9,790	6,026	7,612	.....	.....	.....	.....
Group average		.....	.....	.....	.....	37.9	1.55	30.3	1.23
5	1.0	72,756	2,641	1,771	2,146	41.1	1.49	33.9	1.23
6		20,561	739	640	854	32.1	1.15	24.1	0.87
7		64,919	2,278	1,680	2,089	38.6	1.36	31.1	1.09
8		54,282	2,135	1,748	1,906	31.1	1.22	28.5	1.12
Group total		212,518	7,793	5,839	6,995	.....	.....	.....	.....
Group average		.....	.....	.....	.....	35.7	1.31	29.4	1.08
9	1.5	41,256	1,639	1,131	1,471	36.5	1.45	28.1	1.11
10		83,614	2,978	1,753	2,089	47.7	1.70	40.0	1.43
11		72,051	2,628	1,675	1,906	43.0	1.57	37.8	1.38
12		79,888	3,301	1,874	2,146	42.6	1.76	37.3	1.54
Group total		276,809	10,546	6,433	7,612	.....	.....	.....	.....
Group average		.....	.....	.....	.....	42.5	1.62	35.8	1.37
13	2.0	86,023	3,020	1,787	2,089	48.1	1.69	41.2	1.45
14		77,654	2,897	1,713	1,906	45.3	1.69	40.7	1.52
15		6,604	227	166	198	39.8	1.37	33.4	1.15
16		83,772	2,932	1,835	2,146	45.7	1.60	39.0	1.37
Group total		254,053	9,076	5,501	6,339	.....	.....	.....	.....
Group average		.....	.....	.....	.....	44.7	1.59	38.6	1.37
17	2.5	81,433	3,018	1,799	2,146	45.3	1.68	38.0	1.41
18		44,271	1,674	1,298	1,471	34.1	1.29	30.1	1.14
19		63,298	2,096	1,526	1,906	41.5	1.38	33.2	1.10
20		86,514	2,731	1,813	2,089	47.7	1.51	41.4	1.31
Group total		275,516	9,519	6,436	7,612	.....	.....	.....	.....
Group average		.....	.....	.....	.....	42.2	1.47	35.7	1.24

ficial insemination to purebred Holstein-Friesian bulls. The control group had an average of 2.8 inseminations per conception; the fluorine-supplemented groups were more efficient, 1.5 to 2.2 (table 4).

TABLE 4—The Reproductive Performances of Cows Fed Sodium Fluoride Over a Six-Year Period Period

Total daily fluorine (mg./kg. body wt.)	Av. inseminations per conception	Av. gestation (days)	Alive at birth (%)	Sex of calves
0.15–0.3	2.8	262	75	12 M 8 F
1.0	1.5	259	86	14 M 6 F
1.5	2.2	270	95	8 M 12 F
2.0	1.6	277	100	13 M 5 F
2.5	1.9	272	95	9 M 12 F

The insemination:conception ratios did not appear to correlate with the fluorine treatments.

Average gestation time (270 to 277 days) for cows fed 1.5, 2.0, and 2.5 mg. of fluorine more closely approximated the accepted gestation time of 280 days than did the control group and the group fed 1.0 mg. of fluorine (259 to 262 days). In addition, the over-all viability at birth of the calves in the control group and the group fed 1 mg. of fluorine was only 75 and 86 per cent, respectively. Cows fed higher levels of fluorine had viability rates of 95 to 100 per cent.

Two to 4 calves born to cows in each group were weighed at birth, and all were in the normal range of 90 to 110 lb.

Weight Gains.—A gradual increase in the yearly average weight of each group was recorded for the first five years of the experiment (table 5). During the last year of the experiment, a slight drop in weight occurred for the control group and for the groups fed 1.0 and 1.5 mg. of fluorine. Average weights during the last year for the groups fed 2.0 and 2.5 mg. of fluorine remained at the level attained the previous year. Individual weights of cows within a particular group varied considerably. These animals were weighed only at three-month intervals in order to limit disturbance of milk production. Also, the weight of a dairy cow can vary as much as 25 to 50 lb. from one day to the next, so the general trend in yearly weight averages is probably more significant than individual weights. From the yearly averages, it can be seen that 2.5 mg. of fluorine per kilogram of body weight did not adversely affect the weight or weight gains of dairy cows started on the experiment at 2 years of age.

Biochemical Data.—Analyses of the common blood constituents were carried out at intervals as an added check on the general health of the animals. Fluorine in the amounts fed here did not appear to have any effect on hemoglobin, hematocrit, erythrocyte and leukocyte counts, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular concentration.

At the beginning of the experiment, it was hoped that the level of fluorine excreted in the urine would provide a reliable index of the amount currently ingested. Individual analysis showed that there were considerable variations in voided urinary fluorine from cows consuming the same amount of fluorine. Although this variation was less evident in group averages, the average values for a particular group of cows sometimes varied considerably from one sampling date to another. Conversion of the "as voided" urine specific gravities to a constant value of 1.040 further decreased the range of vari-

TABLE 5—Average Yearly Group Weights (in lb.)

Group	Total daily fluorine (mg./kg. body wt.)	Year and age of animals					
		1950 (2 yr.)	1951 (3 yr.)	1952 (4 yr.)	1953 (5 yr.)	1954 (6 yr.)	1955 (7 yr.)
I	0.15–0.3	1,035	1,098	1,214	1,287	1,376	1,300
II	1.0	1,013	1,102	1,162	1,245	1,338	1,291
III	1.5	1,025	1,068	1,136	1,212	1,297	1,262
IV	2.0	1,000	1,074	1,149	1,180	1,224	1,226
V	2.5	1,043	1,136	1,176	1,279	1,374	1,380
Herd	....	1,023	1,098	1,174	1,244	1,326	1,292

TABLE 6—Urinary Fluorine Excretion of Cows Fed Sodium Fluoride *

Total daily fluorine (mg./kg. body wt.)	Collective summary	
	Average	Range
0.15–0.3	2.6	0.8– 8.8
1.0	12.6	2.6–32.2
1.5	16.1	3.4–30.8
2.0	19.0	6.8–41.1
2.5	25.5	7.5–51.5

* In parts per million corrected to 1.04 specific gravity.

ation between values. Despite these drawbacks, the over-all group averages of urinary fluorine values showed a relative correlation with fluorine intake (table 6).

The levels of fluorine as fed in this experiment had no effect on the specific gravity of the cows' urine, the group averages being in a narrow range of 1.030 to 1.034. Individual specific gravities also were examined with reference to the fall and winter periods of dry feeding and the spring and summer periods when green forage was available. Neither season nor type of feed was found to affect the urine specific gravities.

The usefulness of urinary fluorine excretion data is limited, since the inherent variations make it impossible for urinary fluorine levels to be used as the sole criterion of fluorine intake. However, the average values from several cows of a herd can indicate, in a broad sense, whether they are ingesting fluorine in "normal" quantities (0 to 5 p.p.m. in the urine), in slightly increased concentration not sufficient to cause economic damage (10 to 15 p.p.m.), or at levels expected to cause damage (20 p.p.m. and above).

Examination of the data on fluorine deposition in the bones and cartilage of cows that died or were killed (table 7) shows that fluorine deposition after repeated ingestion occurred in the following decreasing order: rib cartilage, ribs, and jaw and leg bones. At the end of the experiment, the fluorine content of the rib cartilage in cows fed 2.5 mg. of fluorine was 6.4 times that of the controls, and for cows fed 2.0, 1.5, and 1.0 mg. of fluorine, the rib cartilage:fluorine ratios were 6.0, 4.7, and 3.5, respectively, as compared with the controls. The rib cartilage data showed less variation than did the bone values between animals fed the same amounts of fluorine. After almost six years of controlled feeding, the fluorine concentration in the bones of the controls was 440 to 770 p.p.m. of fluorine. For the same period, cows fed 1 mg. of fluorine had 1,930 to 3,000 p.p.m.; 1.5 mg. of fluorine, 2,450 to 4,700 p.p.m.; 2.0 mg. of fluorine, 2,700 to 5,100 p.p.m.; and 2.5 mg. of fluorine, 3,550 to 7,200

TABLE 7—Fluorine Content in the Bones of Cows Fed Sodium Fluoride*

Cow (No.)	Total daily fluorine (mg./kg. body wt.)	Months on experiment	Metacarpal	Metatarsal	Ribs		Mandible	Rib cartilage
					12th	13th		
1	0.15–0.3	70	697	648	762	756	707	941
2		48	400	381	471	448	407	.....
3		62	445	473	575	557	519	808
4		68	560	551	697	730	720	950
5	1.0	70	2,402	2,215	2,640	2,791	2,828	3,201
6		22	.....	.....	.....	2,938	.....	.....
		28	1,050	.....	2,020	.....	1,840	.....
7		68	1,934	2,012	2,509	2,883	2,813	3,373
8		62	2,150	2,366	2,998	2,936	2,865	3,737
9	1.5	48	1,558	1,581	2,757	2,697	3,156	.....
10		22	.....	.....	.....	3,430	.....	.....
		68	2,467	2,608	3,739	3,698	3,474	4,640
11		62	3,343	2,996	4,892	4,722	3,968	6,639
12		70	2,470	2,549	3,822	3,987	3,926	4,282
13	2.0	68	2,719	3,081	4,381	4,386	4,357	5,566
14		22	.....	.....	.....	3,587	.....	.....
		62	3,424	3,402	4,621	4,646	3,951	5,617
15		6	1,040	.....	.....	.....	.....	.....
16		70	3,317	3,372	5,090	4,862	5,081	5,780
17	2.5	70	3,569	3,550	4,651	4,552	4,762	4,582
18		48	3,623	4,119	4,626	4,870	4,035	.....
19		22	.....	.....	.....	5,000	.....	.....
		62	4,337	4,286	6,415	5,826	5,234	5,279
20		68	5,346	5,343	7,210	6,908	6,720	7,439

* On a parts per million, dry, fat-free basis.

p.p.m. of fluorine, respectively. Data obtained from biopsies made 22 months after the start of the experiment and from terminal analyses suggest that fluorine was rapidly deposited in the bones during the first months of the experiment and then only slowly as the bone became more saturated.

Several calves, from cows in each group, were killed at birth and their metacarpal bones removed for fluorine analysis. The data (an average of 3 to 5 values for each group) clearly show that there was a transfer of fluorine from the cow to the fetus and a slight, but gradual, increase in fetal bone fluoride with greater fluorine intake by the mother (table 8). There was

TABLE 8—Fluorine Concentration in Metacarpal Bones of Newborn Calves *

Total daily fluorine fed cows (mg./kg. body wt.)	Av. fluorine in fetal metacarpal bones
0.15–0.3	43
1.0	99
1.5	113
2.0	152
2.5	165

* On a parts per million, dry, fat-free basis.

no correlation between the amount of fluorine in the fetal bone and the period a cow had been used for the experiment.

The soft tissues did not contain appreciable amounts of fluorine. Lung, liver, and heart, on a dry-weight basis, analyzed from 0.4 to 5.0 p.p.m. of fluorine, regardless of the amount fed. Fluorine in the kidney was more variable (1.4 to 25.0 p.p.m.). It is not known, however, if the kidney values represent intercellular fluorine or if they merely reflect the amount of fluorine in the collecting tubules of the kidney at the time of death. The adrenal gland consistently analyzed as high as or higher than any of the other soft tissues, with a maximum value of 44 p.p.m. of fluorine obtained from 1 animal.

Physical Examination.—Periodic examinations of the incisor teeth made it possible to evaluate the degree of dental fluorosis in developing teeth of cows fed known amounts of fluorine. To describe these changes, the following terms were formulated for use in clinical examination of the teeth:

Temporary (Deciduous or Baby Teeth).—So far as is known, temporary teeth in cattle have never shown any markings due to fluorine.

Normal.—As used, this term includes all conditions found in cattle teeth not associated with fluorine lesions. It means normal only so far as fluorine effect is concerned. Actually, many abnormalities are found "normally," and there is great variation due to environment and breeding.

Erupting (The Process of Replacement of the Temporary by the Permanent Tooth).—Although some indication of a fluorine effect may be seen at this stage, an accurate evaluation is not always possible, due to the covering cuticle.

Dull.—This term is applied to a newly erupted tooth that still has a heavy cuticle but which may show definite mottling. At this stage, the tooth reflects a dull cast. The term may also be used to describe an older tooth, when extensive mottling and stains give a dull appearance.

Mottling.—This term is applied to the various markings which occur in the enamel as a result of incompletely calcified enamel rods or poorly developed interprismatic substance or both. It is used here only to denote an effect of fluorine. Other types are called atypical mottling.

Very Slight Mottling (The First Degree of Mottling).—The entire tooth has a normal translucent cast and excellent texture and wearing qualities. The mottling can be readily seen by completely drying the tooth and applying proper light. It is manifested by one or more dense flaky areas that appear to lie deep in the enamel. Often, it can not be seen in a newly erupted tooth, but becomes readily visible after the cuticle wears away. There is some evidence that this type of mottling becomes increasingly difficult to see as the animal ages. There is no unusual wear and, usually, no fluoride stain.

Slight Mottling.—This is a more intense mottling, which gives the tooth a dull, chalky white, or opaque appearance. Part or all of the tooth may be affected. The texture of the enamel is good, and the teeth show little or no unusual wear. Various degrees of staining may accompany a slightly mottled condition.

Marked Mottling.—The teeth are dull, chalky white, or opaque, with a tendency toward yellowish or brownish discoloration. The texture of the enamel is poor, denoting a more severe form of hypoplasia. Excessive wear and enamel erosion are expected to occur.

Stain.—As used here, this term applies only to changes resulting from fluorine intake. It is recorded as very slight, slight, or marked, depending on the amount of staining. A progressive change in the degree of staining within any one tooth has been observed over a period of time.

Two or more of these conditions were often present in a single tooth. The numerical scoring system was thus adopted and used during the experiment. This method of classification is based on a system developed by Dean [3] for human epidemiological studies, with certain modifications. It has been used successfully by Shupe *et al.*[20] and by others in examina-

tions of over 50,000 cattle and sheep. Photographic examples of typical incisor tooth scores are shown (fig. 1).

Explanation of Clinical Dental Scoring System.—The numerical scoring system is described as follows:

Grade 0 (Normal).—This grade includes all conditions found in cattle teeth which are not associated with fluorine lesions. It means "normal" only so far as fluorine effect is concerned.

Grade 1 (Questionable).—This condition may or may not be due to fluorine. It is usually a temporary state and subsequently disappears or increases in severity, allowing a more definite classification.

Grade 2 (Very Slight).—The tooth has very slight mottling and may or may not have yellow or brown staining. The enamel is hard and possesses good wearing quality.

Grade 3 (Slight).—The tooth has slight mottling and a variable degree of yellow to brown stain. The structure is sufficiently sound to prevent subsequent abnormal wear.

Grade 4 (Marked).—The tooth has marked mottling, with various degrees of staining. The structure is sufficiently damaged to permit abnormal wearing. There may be extensive hypoplastic

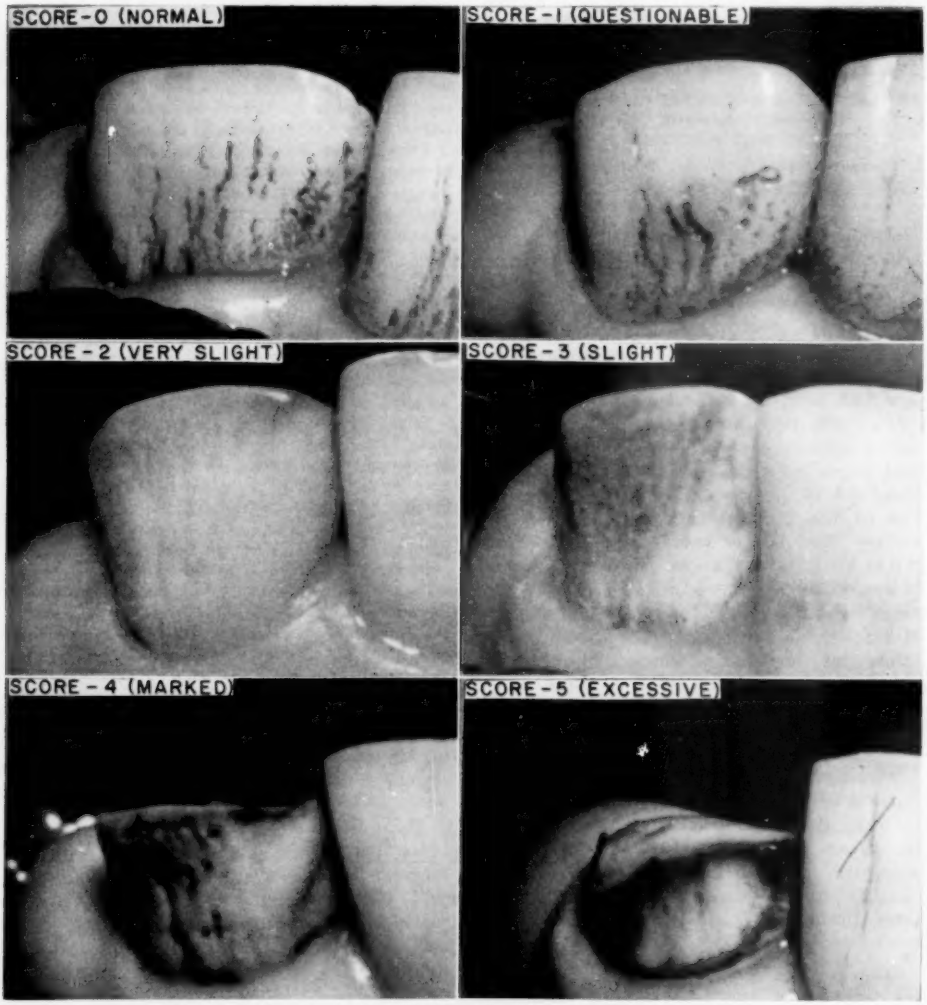

Fig. 1—Incisor tooth classification chart based on fluorine effect.

areas, giving the impression of "pin-point" holes in the enamel.

Grade 5 (Excessive).—This is the same as grade 4 but with increased mottling and extended wear.

No significant changes occurred in the first two pairs of permanent incisors, since they had erupted or were in the process thereof when the experiment started. The third and fourth incisors of cows fed 1 mg. of fluorine showed only slight mottling and staining of the enamel after five years of experimentation. Ingestion of 1.5, 2.0, and 2.5 mg. of fluorine resulted in marked mottling and staining of the third and fourth incisors, with abnormal softening and subsequent wear. Average tooth scores of the cattle after five years on experiment are summarized (table 9). A postmortem photograph of the incisor and molar teeth of cow 18, fed 2.5 mg. of fluorine, is shown (fig. 2). The molar teeth show no abnormal wear.

TABLE 9—Average Incisor Tooth Scores for Each Group of Cows Fed Fluorine as Sodium Fluoride for Five Years, Starting at the Age of 2 Years

Total daily fluorine (mg./kg. of body wt.)	Score for the incisor teeth			
	1st pair	2nd pair	3rd pair	4th pair
0.15–0.3	0	0	0	0
1.0	0	1	2	3
1.5	0	1	2	4
2.0	0	1	2	4
2.5	0	1	4	5

0 = normal; 1 = questionable; 2 = very slight; 3 = slight; 4 = marked; 5 = excessive.

Three cows were killed four years after the experiment started, and their molar and premolar teeth analyzed for fluorine. The values obtained reflected the relative levels of dietary fluorine and correlated well with the bone fluorine values of the particular cows.

Exostotic lesions developed on the metacarpal bones of 2 cows fed 2.5 mg. of flu-

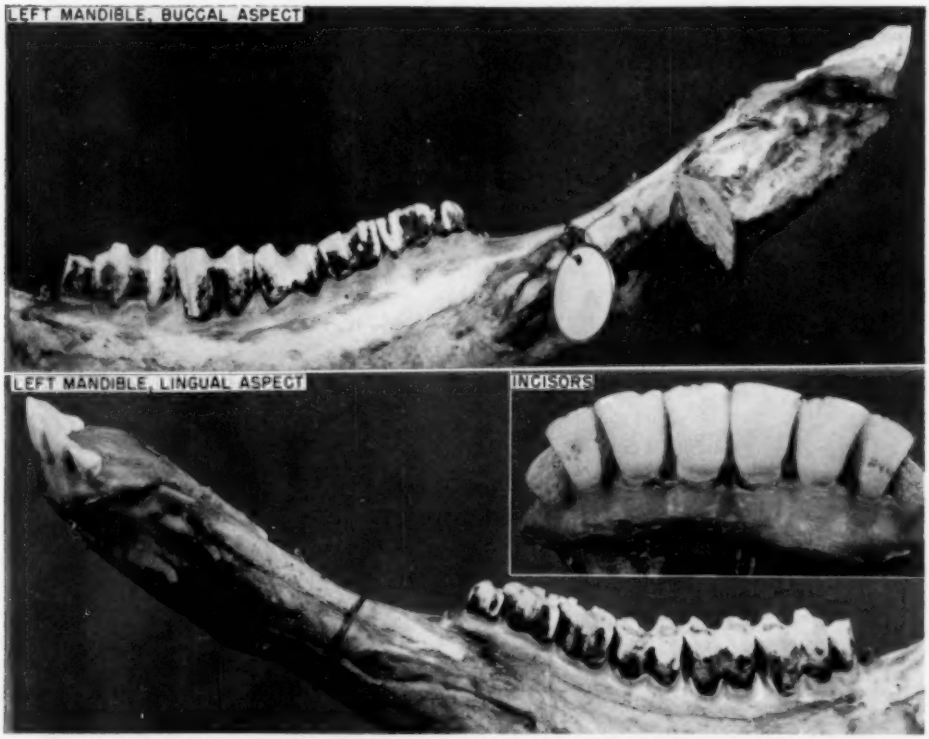

Fig. 2—Incisor and molar teeth of cow 18, fed 2.5 mg. of fluorine per kilogram of body weight per day for four years.

orine. A slight exostosis on the medial surfaces of the metatarsal bones of 1 cow was observed within 16 months. Two months later, another cow in this group had bilateral exostoses of the metatarsal bones. Four months after the exostoses were first observed on the second animal, she showed lameness and general evidence of pain on movement. This condition continued until the end of her second lactation, and recurred intermittently throughout her fourth and fifth lactations.

During the second lactation, cows fed 2.5 mg. of fluorine had a general appearance of unthriftiness. This condition was characterized by a strained attitude, a rougher coat, and a tighter hide compared with those of animals receiving lower levels of fluorine. Occasional instances of unthriftiness also were seen in the group fed 2.0 mg. of fluorine. This condition was most severe when the stress of lactation was at its peak, and then improved or disappeared when the lactation period was completed. The clinical conditions which developed in the cows as a result of continued ingestion of fluorine are summarized (table 10).

Although diarrhea has been described as one of the signs of fluorine toxicosis, this condition was never evident in the animals used for this experiment.

Hoof elongation also has been mentioned as a clinical indication of excessive fluorine

Fig. 3—The periosteal surface and compacta of the metatarsal bone of cow 20. Notice the roughened surface, the increased porosity, and the disappearance of outer basic lamellae. x 57.

TABLE 10—Summary of Clinical Conditions Present After Continued Feeding of Sodium Fluoride to Dairy Cattle

Group	Unthriftiness				Hoof elongation	Diarrhea	Exostoses
	Strained attitude	Rough coat	Tight hide	Lameness			
I	....	....	....	....	....	....	....
II	....	....	....	....	....	....	....
III	10*	9	....	....	....	....	....
IV	13, 16	14	....	....	....	....	....
V	17, 18, 19, 20	18	....	17	....	....	17, 20

* Numbers indicate individual cows.

ingestion. This was investigated by measuring the growth of each cow's left hind hoof over a six-month period during the fifth year of the study. It was clearly demonstrated that there was no significant difference between the groups in net hoof elongation, regardless of the fluorine levels used.

Gross and Microscopic Changes.—The only gross lesions seen on necropsy which could be ascribed to the action of fluorine were slight exostoses on the medial surfaces of the metatarsal bones of cows 17 and 20. These bone proliferations had been observed during clinical examinations four years previously, and did not appear to have increased in size during the ensuing interval.

Four pathologists participated in the histological examinations. Their independent opinions were that the levels of sodium fluoride fed caused no pathological changes in the soft tissues of the cows.

Bone changes were characterized by a roughening of the periosteal surface of the compact bone, and plaques of new bone in the adjacent periosteum of the metacarpal and metatarsal bones and the ribs. Microscopically, these appeared to be a replacement of the outer basic lamella with newly developing haversian canals. As a result, there was increased porosity of the compact bone due to clefts between the haversian systems and increased activity of resorption and replacement of bone in these areas. This suggests that the small dis-

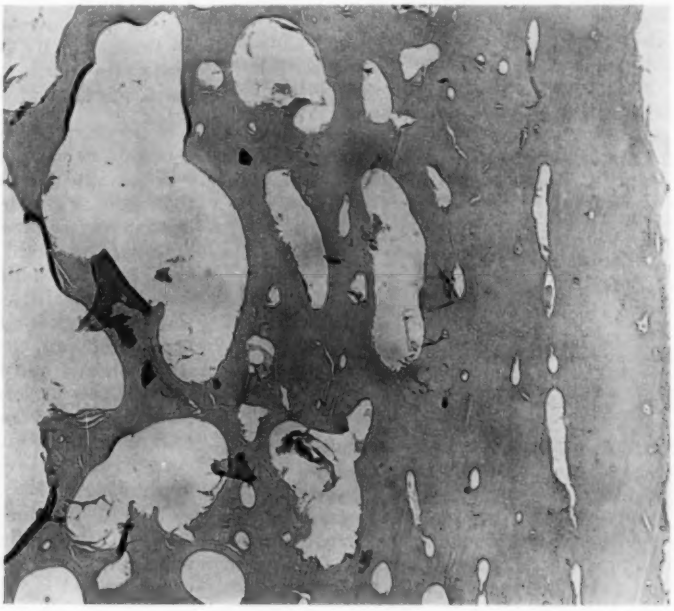

Fig. 4—The compacta of the twelfth rib of cow 20. Notice the irregularity and disappearance of outer periosteal lamellae and the increased porosity. The haversian systems are distorted. x 46.

tortions caused increased activity by osteocytes, osteoblasts, osteoclasts, and blood vessels, which consisted of more rapid resorption and laying down of bone and an increased vascularity, especially in the outer portion of the compact bone and in the periosteum. This represented an attempt by the cells to compensate for the structural deficiency and, unless the intake were extremely high, should have resulted in a bone that was larger and stronger than normal. The increased porosity produced greater strength for the amount of bone. Similar bone lesions in chronic bovine fluorosis have been excellently described by Shupe *et al.*[20] These pathological changes were considered moderate in cows fed 2.5 mg. of fluorine and slight in the group fed 2.0 mg. of fluorine. A few doubtful changes were detected in 2 of the 4 cows fed 1.5 mg. of fluorine. These changes, however, were slight and should not have interfered with the strength or function of the bone to any significant extent.

Relationship of Fluorine as Milligrams per Kilogram of Body Weight to Fluorine as Parts per Million of the Total Ration.—After the feed and water intake of each animal was measured and aliquots of the feed were analyzed for fluorine, the total naturally occurring fluorine consumption was calculated on a parts per million basis. The average intake of naturally occurring fluorine for the herd was 7.4 (±0.5) p.p.m. The average total daily fluorine intake for each group is tabulated (table 11). When

TABLE 11—The Relationship of Fluorine Intake Based on Milligrams per Kilogram to that Based on Parts per Million

Group	Total daily fluorine	
	Mg./kg. of body wt.	p.p.m. in ration
I (control)	0.15–0.3	7.3
II	1.0	33.6
III	1.5	44.7
IV	2.0	60.4
V	2.5	85.6

the experiment was first planned, calculations from data in the literature indicated that a 1,000-lb. dairy cow, consuming a normal ration which contained 30 p.p.m. of fluorine, would ingest approximately 1 mg. of fluorine per kilogram of body weight, and that each 15-p.p.m. increase of fluorine would be approximately equivalent to an increase of 0.5 mg. of fluorine on a body weight basis. The experimentally obtained values in parts per million are in close agreement with the estimated values of parts per million versus milligrams per kilogram.

Under the conditions of measuring feed intake of each animal, the average dry weight of solids consumed daily per 1,000-lb. cow was 28.9 lb. This total was composed of beet pulp (1.4 lb.), grain mix (5.3 lb.), alfalfa hay (17.3 lb.), and corn silage (4.9 lb.). Average water consumption was 110 lb., or 13.8 gal.

The concentration of fluorine in the feces increased in relation to the dietary intake. The average fecal fluorine of the cows fed 1.0 mg. of fluorine was twice that of the controls, and the values for the groups fed 1.5, 2.0, and 2.5 mg. were four times those of the controls (table 12). On

TABLE 12—Fluoride in Feces of Cows Fed Sodium Fluoride

Total daily fluorine (mg./kg. of body wt.)	Fecal fluorine (dry wt., p.p.m.)	Total dietary fluorine in feces (%)
0.15–0.3	19	65
1.0	38	30
1.5	74	31
2.0	76	33
2.5	83	26

a percentage basis, fecal fluorine was greatest for the controls and suggests that much of the naturally occurring fluorine in the feedstuffs was less available than was the more soluble sodium fluoride.

COMMENT

No single factor clearly demonstrated that any of the levels of fluorine used in this experiment could be considered the boundary at which damaging effects to dairy cattle would be expected to occur. A review of all the data suggests, however, that the higher levels of fluorine can be toxic and can cause economic loss. This conclusion is supported by the occurrence of exostoses in cows 17 and 20, lameness and evidence of pain in walking of cow 17, and the appearance of unthriftiness and roughened coats of cows fed 2.0 and 2.5 mg. of fluorine.

Within the limitations of this six-year study, the boundary level at which toxicosis may be expected to occur over periods of prolonged ingestion is 2.0 to 2.5 mg. of

fluorine per kilogram of body weight per day.

SUMMARY

1) The addition of fluorine, as sodium fluoride, at levels as high as 2.5 mg. per kilogram of body weight per day caused no significant difference in milk production between control and fluorine-supplemented animals.

2) The inseminations per conception, days in gestation, and percentage of live births were not influenced by the levels of fluorine used. Birth weights of calves were all within the normal ranges, regardless of fluorine treatment.

3) Fluorine, as sodium fluoride, fed at levels up to 2.5 mg. per kilogram per day did not influence the rate of growth.

4) Individual urinary fluorine values varied widely with the same level of fluorine intake, but group averages tended to show a relationship to the amount of fluorine ingested.

5) Fluorine deposition in bone and cartilage was found to be the most reliable indicator of the levels of fluorine ingested. Relative concentrations were found consistently in the following decreasing order: rib cartilage, ribs, mandibles, and leg bones. Analyses of metacarpal bones of newborn calves demonstrated a slight placental transfer of fluorine from the cow to the calf. Soft tissues did not store appreciable amounts of fluorine.

6) The degree of dental fluorosis was dependent upon the length of time on experiment, the amount of fluorine fed, and upon the age of the cow when sodium fluoride was first administered. Permanent teeth already erupted when the experiment was begun were not significantly affected.

7) The general health of the herd remained on a high plane throughout the experiment. However, frequent appearances of unthriftiness and roughened coats were seen in cows fed 2.0 and 2.5 mg. of fluorine. Exostoses occurred on the metatarsal bones of 2 cows fed 2.5 mg. of fluorine and one of these animals subsequently exhibited occasional signs of pain and lameness in walking. Hoof elongation did not occur as a result of ingesting sodium fluoride.

8) No occurrence of diarrhea was observed which could be attributed to the feeding of sodium fluoride.

9) Bone lesions consisted of roughening of the periosteal surfaces of the compact bones and the presence of plaques of new bone in the adjacent periosteum of the metacarpal, metatarsal, and rib bones. No histological changes which could be ascribed to the ingestion of sodium fluoride were found in the soft tissues.

10) The ingestion of fluorine on a parts per million basis was 7.3, 33.6, 44.7, 60.4, and 85.6 in the feed and water for cows fed 0.15 to 0.3, 1.0, 1.5, 2.0, and 2.5 mg. per kilogram of fluorine, respectively.

11) The results of this six-year study, wherein adult Holstein-Friesian cattle were maintained under practical farm conditions and kept at a high rate of milk production, suggest that 2.0 to 2.5 mg. of fluorine per kilogram of body weight per day is the level at which toxicosis may be expected to occur, after periods of prolonged ingestion.

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Studies on Myoclonia Congenita. I. Review of Literature and Field Investigations

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THE EXISTENCE of a congenital tremor disease in baby pigs has been recognized in this country for at least 35 years, but little authoritative information on this condition is available. This paper reports the principal findings of an investigation of this disease on 42 premises in Minnesota. Later papers will deal with breeding experiments, gross and microscopic pathological findings, and pathological neurophysiology.

The disease has been reported from North America, Europe, and Australia. The distribution within the United States is sketchily known, and no estimates of mortality are available. The cause of myoclonia congenita has not been demonstrated, although a number of theories have been suggested. The disease apparently has not been reproduced experimentally and no successful treatment has been developed. A few reports of pathological studies in small numbers of animals are available. No lesions pathognomonic of this disease have been described.

REVIEW OF LITERATURE

"Dancing pigs" was first described by Kinsley,[12] in 1922, as a condition occurring in pigs under 1 month of age; the signs varied from muscular twitching to uncontrolled movements. In the more severely affected pigs, the feet actually left the ground; thus, the name, "dancing pigs." In 1932, Nissley[16] reported tremor in about half the offspring of 39 sows in one herd. These pigs recovered in two to three weeks and seemed to thrive as well as the unaffected pigs. The disease could not be associated with any 1 boar or group of sows and, supposedly, all the dams were fed an adequate ration. In 1934, Payen and Fournier[17] reported *porcelets trembleurs* in several of 40 litters on one farm. The temperatures of these pigs were normal and, at necropsy, 3 with tremors had no significant lesions. The condition was believed to be hereditary, since no further trouble was experienced after the boar was replaced.

In 1936, Hughes and Hinman[6] observed tremor in the offspring of 2 sows at the California Agricultural Experiment Station. Some of these pigs still had tremor at 6 weeks of age, and some had enlarged joints. The pigs of one litter were notably weak and tended to "spraddle" in the hind limbs. Their tremor was gone in 14 days. Except for 1 severely affected pig, the tremor was present only when the animals were standing. The authors believed that this condition might be similar to an hereditary tremor of chickens.

Also in 1936, Knilans[13] observed tremor in five of 12 litters on one farm. The pigs were trembling when born and became steadily worse for a few days. These five litters were sired by boar 1, the unaffected seven litters by boar 2. All 12 dams were related and 2 were litter mates; only the offspring of the 1 mated to boar 1 showed tremor. Later in the season, 2 sows mated to boar 2 also farrowed "shaky" pigs.

In 1937, Hindmarsh[7] reported on congenital tremor of baby pigs in Australia. Sows 1 and 2 gave birth to litters whose members all suffered from tremor. Gilt 3, a daughter of sow 1, was mated to the sire of these litters and the pigs produced were so seriously afflicted with tremor that all died within seven days. It is not clear whether gilt 3 had tremor when young; she may have been from an earlier (normal) litter. Later, the same boar was again mated with sows 1 and 3 and all the progeny were apparently normal. Consequently, the author concluded that this disease is probably not genetic in origin.

Glässer[4] observed this disease in Germany and compared it to chorea (St. Vitus' dance) in man. However, there apparently is no disturbance of consciousness in pigs. Glässer[4] spoke of a jumpy or jerky limb activity (with flexor movements predominating), which abated when the pig was at rest or asleep. He believed that the tremor was not inherited but was associated with improper protein, vitamin, and mineral supply for the dam during the latter part of gestation. He observed that the 4 sows involved were bred by 2 different boars and that other sows mated with 1 of these boars produced normal litters both before and after the litters affected with tremor. He mentions another sort of tremor in older pigs, which appears as rhythmic superficial muscle contractions only when the animal is lying down. He reported that the tremor of baby pigs could be recognized by its

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appearance when the pig is standing and also by the aggravating influence of cold.

In 1950, Kernkamp[10] reviewed the literature and reported that tremor of baby pigs had been observed at times in the University of Minnesota Agricultural Experiment Station herds over a period of 15 years, but never in more than six of the 30 to 60 litters of the herd, and seldom caused deaths. He suggested that this disease be called "myoclonia congenita."

Lamont *et al.*[14] observed tremor in an entire litter of 12 purebred Saddleback pigs. Twitching was accentuated by excitement or sudden noise. During the first 24 hours, 3 died. The sire of this litter produced offspring with tremor in two other instances, as well as several normal litters. These authors quoted a Professor Biggart, who suggested that this disease might be due to a muscle fiber immaturity, such as that seen in Krabbe's disease in man.

Goodwin[6] investigated the relationship of hypoglycemia to various baby pig diseases and found that, at birth, a number of pigs with congenital tremor had liver glycogen and blood sugar concentrations within the normal range.

Brooksbank[3] investigated "trembles" on nine farms in England. Two or more litters were involved on some premises. The dams were of a wide variety of breeds, but all boars were of the Large White breed. The diet was considered adequate in all but one instance. Affected litters were from both gilts and sows. No lesions were found on necropsy, and bacteriological findings were negative. Swine fever (hog cholera) had been confirmed on five of the nine farms and was, apparently, found within reasonable proximity to the other four farms. Since "trembles" was more prevalent than in previous years, and an epizootic of swine fever had also occurred, this author suggested that swine fever or other viruses might be responsible. The mechanism suggested was that of alteration of neurones of the corpus striatum during a "sensitive period" in their development in the embryo. Since neurotropic viruses were most apt to be involved, he listed the viruses of swine fever, Aujeszky's disease, and porcine encephalomyelitis (Teschen disease) as possible etiological agents.

Larsson[15] reported on a large number of cases of tremor in baby pigs in Sweden. One boar had sired 50 litters with tremor, and many of these pigs died soon after birth. In another instance, a boar which had sired 20 normal litters on one farm was transferred to a location where almost all of his progeny were affected with tremor. Mating a boar and sow which had survived the disease produced a litter of normal pigs. On a third premise, a boar had sired 300 normal litters, while a younger boar, which had been brought in, sired pigs with tremor in ten of 15 litters. Following this, a sow which had previously farrowed a normal litter was again mated twice with the older boar and produced first a litter of pigs with tremor, then a litter of normal offspring. Larsson[15] cited many instances where litters with tremor appeared after a new boar was brought onto the premises. In one, a boar sired 70 normal litters from October 15 to December 17, then after breeding 2 sows which had failed to conceive to another boar, in the next 16 days sired 39 litters, of which at least 17 were affected with tremor. He[15] concluded that tremor disease was not inherited; his only explanation was that it resulted from a virus infection against which individual resistance varied.

Florio *et al.*[5] observed "trembling disease" of baby pigs in France and reported on their necropsy findings and method of treatment. On the basis of the tremor, ataxia, and slight exophalmia in one litter of pigs, they concluded that the basic defect was hyperthyroidism. They reported finding hypertrophic changes in the thyroids of 2 pigs and favorable effects from antithyroid drug administration in 4 pigs. They thought that the dam was a victim of a subclinical virus infection during the gestation period and that this, in turn, caused thyroid abnormalities in the fetus.

After carrying out selective matings among swine which had survived tremor, Goodwin and Palmer[6] concluded that it is not an hereditary trait. They stated that the nerve cells in pigs with tremor appeared normal. However, Hupka and Horn[9] concluded that tremor of baby pigs is transmitted genetically as a recessive characteristic.

Christensen and Christensen[2] have furnished a good clinical description of the typical disease. They thought that errors in feeding the dam during gestation have an important etiological role in myoclonia congenita. They reported that the only characteristic histological finding was a lack of normal myelinization in the spinal cords of pigs with tremor, and that these results were variable.

MATERIALS AND METHODS

The most productive method of locating cases of myoclonia congenita was that of contacting veterinarians by letter. This letter briefly described the typical disease and requested experimental animals for study.

About 80 per cent of the histories were obtained by visits to the farms. A small number of reports were obtained by questionnaires to owners.

RESULTS

Scope.—Myoclonia congenita was investigated on 42 premises. If tremor had been observed in some of the offspring of a certain boar, data were obtained on as many of his offspring as possible, regardless of whether they showed clinical evidence of tremor. When other boars had been used on the same premises and their offspring showed no signs of tremor, they were excluded. Thus, 186 litters on 42 premises were investigated (table 1).

Pigs with Tremor.—Severity of tremor

TABLE 1—Data on 42 Herds Where a Diagnosis of Myoclonia Congenita Was Confirmed

Litters	No.	No. pigs farrowed	Average litter size	Neonatal death loss		Pigs showing clinical tremor	
				(No. pigs)	(%)	(No.)	(%)
Litters showing tremor	128	1096	8.6	230	21.0	796	70.8
Litters showing no tremor	58	416	7.2	75	18.0	.....	.....
Totals	186	1512	8.1	305	20.2	796	52.6

involvement varied extensively between and within litters. Many pigs had such severe tremor that the hindlimbs actually left the ground at each rhythmic twitch of the muscles. Other pigs had less severe tremor and, in some, the fine oscillations of the head and limbs were noticeable only on careful scrutiny. In most litters where tremor was obvious in only a portion of the pigs, careful observation would reveal some degree of tremor in the entire litter. However, in a few litters it could be detected in only a few of the pigs.

The owners usually had observed the tremor at birth or within a few hours after birth. However, one owner first noticed the tremor on the second day, two noticed no tremor until the pigs were 1 or 2 weeks old, and one noticed it when pigs were 3 weeks old.

A few litters were given nutritive supplements or oral glucose, but without benefit. A few owners reported beneficial effects from exposure to heat lamps or to the warmth of the sun.

The disease often appeared to have little or no effect on the baby pig other than the tremor. One owner commented that the affected pigs "seemed to grow better than the others." However, careful observation of the affected litters often showed marked hindleg weakness and perceptible dullness, which seemed to interfere with vigorous nursing activity. This same dullness could, at times, be observed in apparently unaffected litters on the same premises. Members of such litters tended to have a high neonatal mortality.

TABLE 2—Data on Parity of Dams in Herds with Myoclonia Congenita

Litters	Gilts	Sows	Unknown	Totals	Ratio of gilts to sows
Litters showing tremor	90	26	12	128	3.46:1
Litters showing no tremor	27	22	9	58	1.23:1
Totals	117	48	21	186	2.44:1

Dams of Affected Litters.—When dams of unknown parity (table 2) are excluded, there seems to be a greater tendency for tremor to appear in the offspring of gilts than of sows.

The nutrition of dams of myoclonic litters appears to have been adequate in most instances. Of 28 herds on which information was available, 21 were kept on good pasture, such as brome, alfalfa, Ladino, or bluegrass, during the summer months. Most of the rations included corn, oats, alfalfa, meal, and mineral and protein supplement. In no instance was it apparent that dams farrowing trembling pigs were fed differently from other dams on the same premises which farrowed normal litters.

According to the owners, the health of the dams of trembling pigs was good in most instances (table 3). In eight herds, the chief complaint was a general stiffness and, occasionally, a localized lameness.

TABLE 3—Health of Dams

Owners' comments	No. of herds
Good health	28
Some were lame, especially just before farrowing	1
Stiffness after weaning	1
Stiffness and lameness both before and after farrowing	3
Poor appetite near end of gestation and after farrowing. A few seemed stiff earlier in the winter. Also poor lactation	1
Several had difficulty farrowing. Poor appetite. Five of 13 were sick and 2 died	2
No health history	6
Totals	42

Of the 27 affected herds in which histories on hog cholera vaccination were available, the dams had been vaccinated with serum and virus in six and modified live virus in five. There was no vaccination against hog cholera in the other 16 herds. Hog cholera had occurred in three of the herds investigated, but not in the last 15 years.

Dams of affected litters included Berk-

shire, Chester White, Duroc-Jersey, Hampshire, Landrace, Minnesota (No. 1, 2 and 3), Spotted Poland China, Montana, Yorkshire, and various cross breeds.

Sires of Affected Litters.—With few exceptions, the myoclonic litters were sired by purebred boars. With the addition of the Poland China breed, and excepting the Spotted Poland China breed, the sires were of the same breeds as the dams.

Most of the sires were, apparently, in good health. Since many were owned in partnership with one or more neighbors, a long-term health history was difficult to obtain. The chief complaint (table 4) was stiffness and lameness.

TABLE 4—Health of Sires

Owners' comments	No. of herds
Good health	14
Boar purchased at 3 months of age. Did not grow well; seemed dull and would not defend himself against the other pigs	1
Temporary lameness on at least two occasions	1
Possible rhinitis	1
Fever and bronchitis the previous fall. Now (Feb.) walks stiffly and favors one hindleg	1
Became thin after he was purchased. Stiff and walked as if feet were sore. Poor appetite	1
No health history	7
Total	26

The 26 boars sired 186 litters, of which 128 were affected with myoclonia congenita. The remaining 58 litters appeared normal. The progeny records obtained for each boar were complete only with respect to the farm or farms being investigated and to litters farrowed before the visit.

In most instances, the boar was newly acquired and had not been raised on the premises. Ordinarily, the boar is purchased from unrelated stock and used to sire one or, rarely, two farrowings of pigs before he is sold to another breeder or sent to market. On several of the farms where myoclonia congenita was present, more than one boar had been used. In none of these instances was more than 1 boar siring myoclonic pigs.

Incidence of Congenital Tremor or Other Neurological Disturbances on 42 Premises in Previous Years.—The owners of 36 herds could recall no unexplained tremor or neurological disturbances in previous farrowings. Reports on the remaining six herds are detailed.

E. H. Farm.—In 1955, about 50 baby pigs showed tremor, but 3 pregnant sows purchased from a neighbor farrowed normal pigs. The gilts from the normal litters and a gilt which had tremor as a baby pig were then mated (in 1956) with a new boar and all produced normal offspring. One pregnant sow, purchased (in 1956) from the same neighbor, produced offspring with tremor.

M. K. Farm.—In 1954, a Hampshire boar sired several trembling litters. In 1955, a Montana boar was used and even more trouble with tremor resulted. In 1956, a Berkshire boar was used and 12 of 23 litters were affected.

A. K. Farm.—In three consecutive years, the owner purchased bred sows or gilts from M. K. In 1954, pigs in the litters of all 4 gilts showed tremor; in 1955, the 2 sows farrowed normal litters; but in 1956, 2 of 3 sows produced pigs with tremor.

W. L. Farm.—In the summer of 1956, some of the spring pigs had trouble getting up and walked stiffly. They seemed normal after two weeks to a month.

E. N. Farm.—In 1953, a Spotted Poland China boar sired offspring with tremor. (Owner observed "quivering" in this boar.) In 1954, a Minnesota No. 1 boar was used on gilts (which had shown no tremor) farrowed in 1953. The offspring showed some signs of tremor. In 1955, a Yorkshire boar was used on gilts farrowed in 1954; the offspring showed severe tremor.

E. F. Farm.—In 1955, hind leg weaknesses and some tremor was noticed in the baby pigs.

With a few notable exceptions, it appears that myoclonia congenita tends not to recur on the same farm.

Geographical Distribution of Myoclonia Congenita in Minnesota.—Data on the distribution of this disease in Minnesota over approximately a two-year period (fig. 1) are not complete, but represent cases reported by the practicing veterinarians. The total was greater than 42, but time did not permit a detailed investigation of all the affected herds reported.

The disease occurred mainly in the central and southern portions of the state, where the swine population is greatest.

Hereditary Aspects.—The possibility of a genetic factor in this disease has been refuted by other authors.[2,4,7,15] However, one author[9] concludes that the disease is genetically transmitted as a recessive.

The results of this investigation provide no basis for postulating a genetic factor. One boar sired 21 litters with tremor, on eight different farms, from dams representing at least four genetic backgrounds. Another boar sired normal litters at the spring farrowing and, when remated to 2 of the sows, produced litters with tremor at the

fall farrowing. Of the 26 boars involved in the 42 herds investigated, only 1 was raised on the premises where he was used; the other 25 were purchased from unrelated stock.

The sex of baby pigs with tremor was recorded in 17 litters. Of 88 pigs with tremor, 42 were males and 46 were females.

Seasonal Incidence.—Cases of myoclonia congenita were recorded over a two-year period in all months of the year except July and November. Four herds were affected in January, nine in February, five in March, 12 in April, seven in May, two in June, three in September, and one each in August, October, and December.

DISCUSSION

A total of 80 cases of this disease, involving approximately 3,000 baby pigs, was reported over a two-year period. There are indications that this may reflect as little as 10 to 15 per cent of the true incidence.

Fig. 1—Incidence of myoclonia congenita in Minnesota (May, 1955, to May, 1957). The numerals represent affected premises; no cases were reported in the part of the state deleted on the map (northeast corner). The incidence shown is estimated to represent 10 to 15 per cent of the total.

Most of the data on mortality (table 1) refer to baby pigs which were either born dead or died a few days after farrowing. Therefore, the figures on neonatal death loss represent minimum values. Large neonatal death losses were associated with myoclonia congenita in some instances, but not in others.

Data obtained from Kernkamp [11] on a large number of litters farrowed over a nine-year period show a baby pig mortality, from all causes, of 20.8 per cent during the first seven days of life. By comparison, the 21.0 per cent (table 1) neonatal mortality (in litters with tremor) has no significance. However, the cause of this high mortality among baby pigs remains undetermined and this disease could be a factor.

The incidence of affected litters was considerably greater among gilts than among sows (table 2). This might be because a dam carrying her first litter may still be growing and, therefore, may be more susceptible to nutritional deficiencies than a sow. However, on farms where the disease occurred in the offspring of only some of the dams, it was not possible to segregate these dams on the basis of nutrition, but a sharp line always could be drawn on the basis of the sire used. Where more than 1 boar had been used, the disease occurred only in the offspring of the most recently acquired boar. This does not imply that the disease can not occur in the offspring of more than 1 boar in a given situation, since the opposite has been reported.[2, 15]

A more probable interpretation of the higher gilt to sow ratio, as dams of litters with tremor, is that the gilts may lack resistance to whatever agent may be causing the disease. It has been suggested [15] that this disease may result from a virus infection and that resistance varies with the constitution of the individual animal.

Brooksbank [1] has emphasized the significance of hog cholera (swine fever) infection on the same or adjacent farms in connection with the occurrence of "trembles." However, none of the data obtained in this investigation directly or indirectly support this contention. It was also found that myoclonia congenita appeared in the offspring of both vaccinated and unvaccinated dams.

Some of the earlier observations [15] made on this disease indicated that it might occur only in certain breeds. The present study

shows that the disease occurs in the offspring of a wide variety of breeds, with respect to both sires and dams.

In affected herds where health histories of the breeding stock were obtained, over 20 per cent of the owners reported evidence of illness in either the sire or some of the dams. The significance of such a finding might be questionable if it were not for the prevalence of the syndrome of stiffness and lameness among parents of affected pigs. Most of the owners reported that these signs were transient and, as such, might have been overlooked. Therefore, this might indicate that some common disease process of a generally mild nature may have been present in the parents of myoclonic pigs.

Sex does not seem to be a factor in the occurrence of myoclonia congenita.

From the information on seasonal incidence, there appears to be a greater tendency for this disease to appear in late winter and spring than during the rest of the year. However, this apparent increase probably parallels the increased number of pigs farrowed during this period.

SUMMARY

A field investigation of myoclonia congenita of baby pigs has been conducted in 42 affected herds over a two-year period. This seems to be a fairly common disease entity; the 42 instances investigated probably represent 10 to 15 per cent of all cases. The incidence was considerably greater among the offspring of gilts than of sows. The data give no support to the theories that this disease is associated with inadequate nutrition or with hog cholera. The breed and sex of the pigs did not seem to be factors. There appears to have been some mild disease process, predominantly a stiffness and lameness, among the parents of affected pigs in at least 20 per cent of the affected herds. It is suggested that the sire plays a significant role in the transmission of this disease and that the disease is not genetically transmitted, but is of an infectious nature.

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Studies on Myoclonia Congenita. II. Gross and Microscopic Pathology

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MYOCLONIA CONGENITA is a tremor disease of newborn pigs. A previous paper [16] dealt with a review of the literature and the clinical picture.

REVIEW OF LITERATURE

Payen and Fournier [14] necropsied 3 pigs showing tremor and found no lesions. Hughes and Hinman [8] observed enlarged joints in some pigs affected with tremor. Lamont *et al.*[10] reported that necropsy of 1 pig showed normal viscera, brain, and spinal cord, with no demyelinization or loss of nerve cells in any part of the nervous system. Brooksbank [2] mentions a personal communication from J. T. Done which states that, in affected pigs, the cells of the corpus striatum show degeneration, loss of shape, and vacuolation.

Turbes *et al.*[18] have observed perivascular pigment infiltrations in portions of the cerebellums of myoclonic pigs. At times, these authors had also found some "amorphous" degeneration of the Purkinje cells. Goodwin and Palmer [7] report that, so far in their observations, the nerve cells of myoclonic pigs seemed normal. Christensen and Christensen [3] studied 9 pigs with tremor and found what they thought to be a characteristic lesion. In 3 of these pigs, they found a marked absence of normal myelinization throughout the central nervous system. In 5 of the trembling pigs, they found slight to moderate reduction of myelinization in the lateral tracts of the spinal cord, and in 1 pig with tremor they found apparently normal myelinization.

Menges *et al*[13] describe the lesions of sporadic bovine encephalomyelitis as an intense vasculitis, having diffuse distribution throughout the vascular tree. The endothelium of the blood vessels is described as showing a proliferative change, along with infiltration of the vessel wall with mononuclear cells. Occasionally, polymorphonuclear cells were said to predominate. In addition, the serous membranes are consistently involved by a serofibrinous exudative process. The encephalomyelitis associated with the disease is not primary, but arises as an incident in a widespread invasion of mesenchymal tissues by a virus.

In 1957, Jones *et al.*[9] reported the lesions of equine viral arteritis. The prominent gross findings were edema, congestion, and hemorrhage. These lesions were related in both intensity and frequency with specific microscopic changes in the small arteries, whose diameters usually were 0.5 mm. or slightly larger. The prominent findings were necrosis of the smooth muscle cells, replacement of the media by a homogeneous eosinophilic material, and infiltration of the media by lymphocytes. Associated with these lesions were edema and leukocytic infiltration of the adventitia. Some of the arteries were surrounded by lymphocytes and edema and appeared to be tortuous in their course.

MATERIALS AND METHODS

Detailed necropsy procedures were carried out on 46 pigs. Specimens from each of the anatomic systems of the bodies of some of the animals were collected and examined and, in others, the examination was limited to special organs. Particular attention was given to the nervous and cardiovascular systems. The animals with tremor either had field cases or were from litters farrowed as part of the breeding experiments. Stillborn pigs from litters showing tremor were examined. The control animals were purchased from clinically nonaffected litters. The majority of these pigs were less than 10 days old, since it is during the first few days of life that the tremor usually is most severe.

The tissues were collected at necropsy with a minimum of delay. Nervous tissue was preserved by immersion in 10 per cent neutral formalin. Bouin's fixative was used for the pancreas, and most of the other tissues were fixed by immersion in mercury-formol-saline solution. Tissues which were to be stained for fat were fixed in calcium-cadmium-formol solution.[1] Routine hematoxylin and eosin staining was used on almost all tissues. In addition, sections of the same tissues were stained by means of Crossman's modification of Mallory's triple stain.[5] Nervous tissue also was stained by the Nissl technique.[4] Other specialized staining methods used were the Weigert myelin sheath stain,[12] Holmes' silver stain [11] for nerve fibers, Swank-Davenport stain [17] for degenerating

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Fig. 1—Elastic artery in the capsule of the pancreas of a pig. The internal elastic membrane (1) covers the proliferated tissue (2) which has filled the lumen of the artery. Gömöri's aldehyde fuchsin stain; x 176.

myelin sheath, and Gömöri's aldehyde fuchsin stain [15] for elastic fibers.

RESULTS

The prominent gross lesions were edema, thickening, and hemorrhage in the transverse sinus region of the cerebellar dura, and variable degrees of congestion and hemorrhage at sites such as the brain,

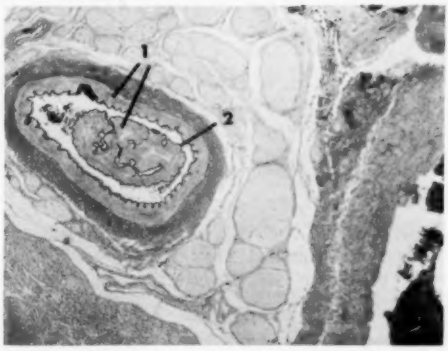

Fig. 2—Elastic artery in the pancreas of a pig. Notice the elastic tissue (1) in the media of the vessel and in the intra-arterial polyp, and the internal elastic membrane (2) covering the intra-arterial polyp. Gömöri's aldehyde fuchsin stain; x 32.

lymph nodes, liver, kidney, extrinsic ocular muscles, lung, spleen, and thymus. Changes at one or more of these sites were observed in over 90 per cent of these pigs.

The most extensive microscopic changes were in the blood vessels of the pancreas (fig. 1, 2). The adventitia of the arteries was thickened by edema, fibrin, collagenous fibers, and cellular infiltration. The cellular infiltration was composed of mononuclear cells, fibroblasts, and macrophages, with an occasional polymorphonuclear cell present (fig. 3). Hyalin-like material was

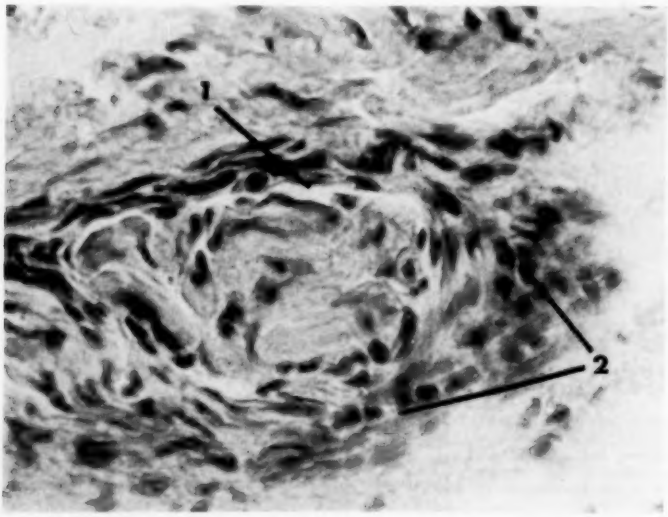

Fig. 3—Small artery in the striated muscle of a pig's tail. The lumen (1) is nearly obliterated, due to a proliferative process. Notice the cellular infiltration of the media and adventitia (2). H & E stain; x 500.

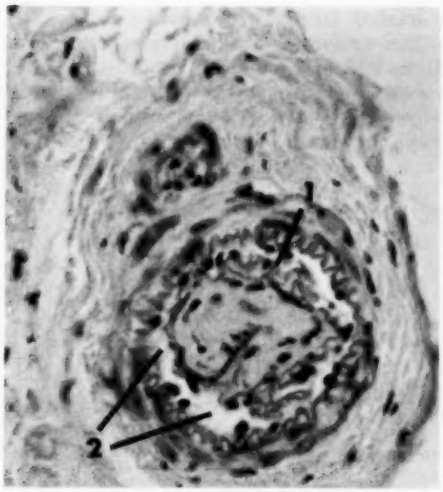

Fig. 4—Small elastic artery in porcine dura. Notice the internal elastic membrane (1) pushed into the lumen (2) by proliferative tissue. Gömöri's aldehyde fuchsin stain; x 240.

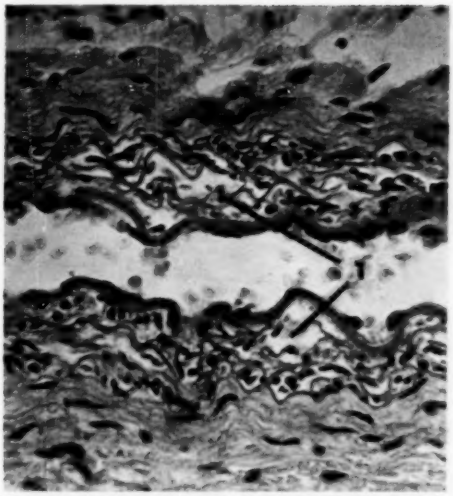

Fig. 5—Elastic artery in the capsule of a pig's thymus. Notice the degeneration of the cells in the media (1), with the elastic tissue remaining. Crossman's modification of Mallory's triple stain; x 400.

observed in the adventitia of some arteries and fragmented nuclei frequently were associated with it. The process involved either the entire circumference of the vessel or only a portion of it. If the vessel was dilated and the wall thin, the adventitia was entirely absent around that part of the vessel in some instances.

The media was thickened by cellular infiltration, hyalin-like material, or a proliferative process which sometimes extended into the lumen (fig. 1, 2, 4). The proliferated tissue appeared to be composed entirely of the cells of the media in most of the vessels and, in others, it was at least partly composed of an infiltration of mononuclear cells and fibroblasts. In still other vessels, the cytoplasm of some of the cells in the media was vacuolated or degener-

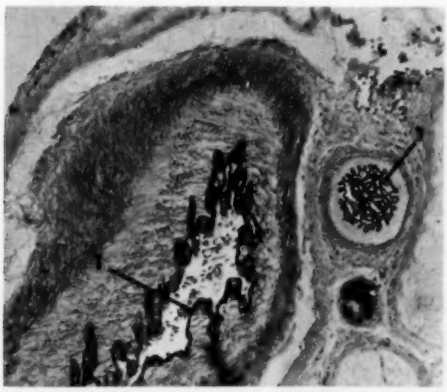

Fig. 6—Vessels in the capsule of the pancreas of a pig. Distorted internal elastic membranes are shown in two arteries (1), with the near obliteration of the lumen in the smaller artery. Gömöri's aldehyde fuchsin stain; x 149.

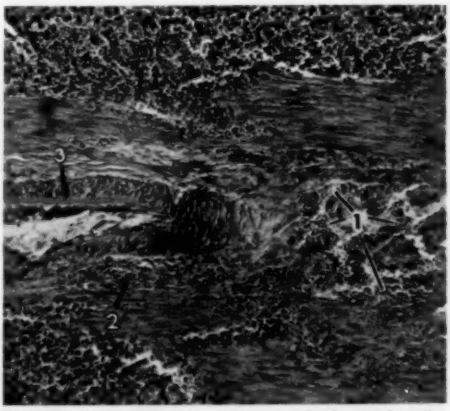

Fig. 7—Artery in a porcine splenic trabecula. There is hemorrhage in the trabecula (1) and surrounding the artery (2). Notice the thickened fragmented internal elastic membrane (3). H & E stain; x 120.

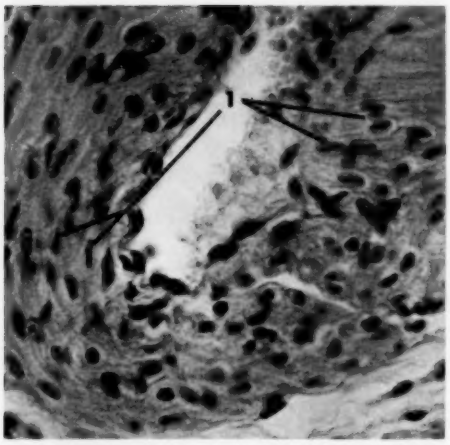

Fig. 8—Small vein in the heart of a pig. Notice the Anitschkow cells (1) in the cardiac muscle surrounding the vein. H & E stain; x 445.

ated, with only pyknotic nuclei and elastic tissue remaining (fig 5). In some of the elastic arteries, the proliferative process of the media displaced the internal elastic membranes into the lumen of the vessel, where it either partially or completely occluded the vessel (fig. 6). The same type of proliferative process of the media was observed in the veins, and it either decreased the size of the lumen or obliterated it completely. No recanalization of these vessels was observed.

In some vessels, the internal elastic membrane was thickened and fragmented, and it stained deeply with eosin (fig. 7). The intima was intact in some vessels, while in others it was desquamated. In still other vessels, the intima was cellular, with enlarged nuclei and little cytoplasm. Hemorrhage into and outside of the wall frequently was seen in the damaged vessels.

Similar but less extensive changes were found in the blood vessels of the heart, spleen, kidneys, lungs, lymph nodes, dura mater, thymus, thyroid, adrenals, gastrointestinal tract, and tails. The tails examined were those which had been undergoing necrosis. In the heart, Anitschkow's cells were observed in the media of some of the smaller branches of the coronary vessels and beneath the endocardium (fig. 8). Edema, fibrin, macrophages, and mononuclear cells were present in the endocardium and, to a lesser extent, in the epicardium; some of the macrophages were vacuolated. Occasionally, hyalin-like material was observed beneath the endocardium.

The most consistent changes observed in the liver were fatty metamorphosis, atrophic hepatic cords, and sinusoids filled with erythrocytes. Vascular changes similar to those described previously also were observed. A large percentage of the lymph nodes showed prominent germinal centers, an increase in the number of lymphocytes, and numerous eosinophils and plasma cells. Occasionally, a lymph node was infiltrated with polymorphonuclear cells. Other frequent findings were hemorrhages into most

Fig. 9—Small muscular artery in the dura of a pig. The lumen (1) is nearly obliterated by a proliferative process (2). Crossman's modification of Mallory's triple stain; x 500.

or all of the alveoli within some lobules of the lung, and plasma cell and eosinophil infiltration into the stroma and follicles of the thymus and spleen.

The dura frequently was affected by edema and hemorrhage. In addition to the vascular lesions in the dura (fig. 4, 9), there often were clusters of mononuclear cells and, occasionally, groups of plasma cells and eosinophils.

Detailed microscopic examination of selected portions of the central nervous systems, using a variety of staining techniques, in 20 animals did not reveal changes which were thought consistent enough to include as part of the pathological picture. Studies of interrupted serial sections of the brain in 3 pigs also failed to reveal any consistent pathological changes.

DISCUSSION AND SUMMARY

The gross pathological changes indicated that vascular damage was probably the basic primary lesion in this disease, with the implication that the nervous system was involved secondarily. It should be noted that this relationship of a primary vascular lesion with a secondary involvement of the nervous system is not a new disease pattern. Sporadic bovine encephalomyelitis is an example of a viral disease with primary vascular lesions, the nervous system being affected secondarily.[13]

The similarity of the vascular lesions of myoclonia congenita to the vascular lesions of equine arteritis is salient. Isolation of a filterable agent causing equine arteritis[6] tends to support further the work now in progress on the isolation of an infectious agent from pigs with myoclonia congenita.

The most consistent microscopic change found in myoclonia congenita was a widespread vasculitis involving the smaller arteries of the vascular tree and, occasionally, affecting some of the smaller veins. The lesion was an inflammatory, degenerative or, occasionally, a proliferative process, with partial or complete obliteration of the lumen. The process involved either part or all of the vessel wall and appeared to be segmental in nature. Ruptured vessels were observed at numerous sites throughout the body, and hemorrhage, edema, and congestion of the tissues involved were noticed frequently.

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Experimental Leptospirosis. IV. Pathogenesis of Porcine *Leptospira Pomona* Infections

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SEROLOGICAL SURVEYS indicate that leptospirosis (*Leptospira pomona* infection) is or has been present in 3 to 25 per cent of the swine in the United States.[2, 3, 5, 27] *Leptospira pomona* antibodies have been found in random sample lots of both commercial anti-hog cholera serum [5, 9] and normal porcine *gamma* globulin.[18] Infected swine represent the greatest potential source of the disease for other livestock, as well as for man.[12, 23, 25] Large numbers of leptospiras are excreted in porcine urine, and the renal carrier state has been found to persist for one year.[22] An experimentally infected hog shed *L. pomona* for 159 days,[5] and 9 of 10 gilts remained urinary shedders for at least six weeks.[4]

The investigations reported here deal with the course in and effect of leptospirosis upon feeder-type hogs, with hematological alterations, and factors influencing the degree and duration of leptospiremia and leptospiruria.

MATERIALS AND METHODS

Duroc-Jersey or Chester White swine (30) were used as experimental animals. Before the beginning of the experiment, their serums did not contain antibodies for *L. pomona*.

The modified agglutination-lysis test [15, 16] was used, employing living *L. pomona* (strain Johnson) cells as antigen and serum dilutions of 10^{-1} through 10^{-8}. The tubes were incubated in a thermostatically controlled water bath for two hours at 37 C. and examined at x 100, using a microscope fitted with an Abbe condenser into which a star diaphragm was fitted. This produced a modified dark-field type of illumination which proved highly satisfactory for reading the tests. Urine leptospiral agglutinins were detected using the same technique, except that the urine was diluted 1:5, 10^{-1}, 10^{-2}, and 10^{-3}. The degree of agglutination, lysis, or both was recorded as 25 or 50 per cent. Such a distinction can be made and agreement among several observers is possible on this basis.

When approximately 3 months old, 13 of the 30 pigs were inoculated subcutaneously with 2 cc. of infected guinea pig blood containing *L. pomona* (strain Wickard) of bovine origin.[14] This Leptospira had been maintained in continual guinea pig passage since its isolation in 1953. Of the 13 pigs, 10 were maintained for a feeding trial to assess the effect of leptospirosis upon swine being raised for market. Ten normal, noninfected litter mates were housed in separate, isolated quarters and served as controls. The pigs were kept in 10- by 13-ft. concrete-floored rooms, with 5 animals in each room. A standard ration was fed ad libitum, and ample fresh water was provided during the 115-day feeding trial. The ingredients and respective percentages of the feed were as follows: corn, 79.6; soybean oil meal, 12.0; meat scrap, 4.0; alfalfa meal, 3.0; limestone, 0.3; dicalcium phosphate, 0.5; trace mineral mixture, 0.5; and vitamin concentrates, 0.7. Weight gains and feed consumption were measured each week.

Three normal pigs (4968, 4958, and 4974) were housed similarly to those on the feeding trial, but were kept in contact with 3 infected pigs. (Leptospirosis was transmitted after approximately one month of contact.)

Rectal temperatures were taken daily. Approximately 0.1 cc. of blood from each pig given the bovine *L. pomona* strain was inoculated into each of five tubes of modified Chang's medium.[6, 14] Cultures were made from most of the hogs on postinoculation days 6 through 9, and were incubated at 30 C. Examinations for the presence of leptospiras were made by dark-field microscopy (x 590) after 14 and 28 days. Modified microscopic agglutination-lysis tests [15, 16] were conducted on serums which were obtained at weekly or monthly intervals. Whenever possible, urine samples were obtained for agglutination-lysis tests, dark-field microscopic examinations for the presence of leptospiras, and animal inoculations, as well as for pH and albumin determinations.

When 7 months of age, 4 of the control hogs (4927, 4928, 4929, and 4973) were inoculated subcutaneously with 5 cc. of blood from infected guinea pigs. The *L. pomona* strain used was obtained from porcine urine by workers at the Ohio Agricultural Experiment Station, Wooster. This strain had been maintained in continual hamster or guinea pig passage since its isolation six months previously. Blood samples were obtained daily on days 1 through 10 from these 4 hogs; cultures were made and serum titers determined.

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The blood cultures were examined by dark-field microscopy (x 590) four times at ten- to 12-day intervals. These frequent examinations indicated relative numbers of leptospiras present in the original 0.1 cc. of inoculum. The erythrocyte, hemoglobin, blood nonprotein nitrogen, reticulocyte, and leukocyte levels, as well as differential leukocyte counts, were ascertained by standard methods [26] on days 1 through 14. Urine samples were collected as frequently as possible for these examinations.

Individual hogs were killed and necropsies performed at 35, 49, 76, 102, 121, 146, 151, 184, and 214 days after inoculation or exposure. Samples of both kidneys from each hog were obtained and homogenized in 0.85 per cent sterile sodium chloride solution to give a 10 per cent tissue suspension. Two to 3 cc. was inoculated intraperitoneally into each of 4 or 5, 250-Gm. guinea pigs, or 1 cc. was inoculated intraperitoneally into each of 3 to 5 hamsters, 4 to 5 weeks old. The bovine strain of *L. pomona* was not lethal for guinea pigs or hamsters, while the porcine strain was lethal only for hamsters. Therefore, kidney suspensions from hogs infected with the bovine strain were inoculated into both species of laboratory animals, while only guinea pigs were inoculated with tissues containing the porcine strain. The inoculated animals were maintained in separate cages, each cage containing only those individuals which received the same inoculum. The original inocula were considered to have contained leptospiras if the serums of the animals contained antibody for *L. pomona* at a dilution of 10^{-2} or higher three to four weeks after inoculation.

RESULTS

Of the 13 pigs inoculated with the bovine strain, 4 showed mild depression on post-exposure day 7 or 8. The animals were reluctant to move, and inappetence was observed. One pig had diarrhea on day 9. Rectal temperatures of 9 pigs were 104 F. or higher during postinoculation days 7 to 10, and 1 had a marked pyrexia, i.e., 105.4 F. Temperatures of 104.0 to 104.8 F. occurred in 5 pigs for one day, in 2 for two days, and in 2 for three days. Temperatures of 104.0 F. for one day occurred in 2 of 3 pigs infected by contact exposure.

The 4 hogs infected with the porcine strain did not show significant febrile responses, but 2 showed depression and 3 a transient, mild diarrhea between days 2 and 8.

In the 4 hogs infected with the porcine strain, preinfection erythrocyte counts ranged from 5,440,000 to 6,240,000; the median for eight samples was 5,940,000. Preinfection hemoglobin values ranged from 10.4 to 12.5 Gm./100 cc. of blood; the median value was 11.4 Gm./100 cc. of blood. For 14 days after inoculation, both erythrocyte and hemoglobin levels remained within normal limits except in animal 4973, which had 4,590,000 erythrocytes and a hemoglobin level of 9.4 on day 8.

Two hogs (4973 and 4982) had slight anisocytosis on days 9, 10, or 11, and had reticulocyte levels of 1.0 and 1.9 per cent on days 10 and 13, respectively.

The total and differential leukocyte counts during the first 14 days did not show significant changes attributable to leptospirosis. The nonprotein nitrogen blood values were within normal limits.

Albuminuria was present during days 12 and 13, but was not demonstrable on day 27 or upon subsequent examinations. The urine did not appear to contain blood but, unfortunately, chemical tests were not conducted to ascertain the presence of occult hemoglobin.

TABLE 1—Correlation Between Leptospiremia and Development of Leptospiral Serum Antibody in Hogs Infected with the Porcine Strain of Leptospira Pomona

	Period of leptospiremia *											Serum antibody titers						
	Days †											Days †						
Hog	1	2	3	4	5	6	7	8	9	10		6	7	8	9	10	11	12
4929	−	−	−	−	+	+	+	−	−	−	A–L 25%**	0§	10^{-1}	10^{-4}	10^{-3}	10^{-4}	0	10^{-7}
											A–L 50%‡	0	0	10^{-3}	10^{-2}	10^{-3}	10^{-7}	10^{-6}
4973	−	−	−	+	+	+	+	+	−	−	A–L 25%	0	10^{-1}	10^{-3}	10^{-4}	10^{-4}	10^{-7}	10^{-6}
											A–L 50%	0	0	10^{-2}	10^{-1}	10^{-3}	10^{-6}	10^{-4}
4928	−	−	−	+	+	+	+	+	+	−	A–L 25%	0	0	10^{-4}	10^{-4}	10^{-4}	10^{-6}	10^{-5}
											A–L 50%	0	0	10^{-2}	0	10^{-3}	10^{-5}	10^{-4}
4929	−	+	−	+	+	+	+	+	+	+	A–L 25%	0	0	10^{-3}	10^{-4}	10^{-3}	10^{-6}	10^{-6}
											A–L 50%	0	0	10^{-1}	0	10^{-3}	10^{-5}	10^{-4}

* Blood (0.1 cc.) cultured in each of five tubes of Chang's medium; † days after subcutaneous exposure; ** A–L 25% = highest dilution showing 25 per cent agglutination or lysis; ‡ A–L 50% = highest dilution showing 50 per cent or greater agglutination or lysis (for example, a serum listed as A–L 25% at 10^{-4} and A–L 50% at 10^{-1} would indicate 50 per cent or greater agglutination at the 10^{-1} dilution, but 25 per cent agglutination at 10^{-2}, 10^{-3}, and 10^{-4}); § 0 = no agglutination or lysis at 10^{-1}.

TABLE 2—Comparison of Serum and Urinary Antibody Titers for 12 Months After Exposure

Time	No. of serums with titers* of									No. of urine samples with titers* of				
	0**	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	0**	0.5×10^{-1}	10^{-1}	10^{-2}	10^{-3}
1 week	7	0	8	2	..	..	..	..	..	1	2	..	..	..
2 weeks	..	..	..	..	2	2	1	..	..	2	1	5	..	..
3 weeks	..	..	..	..	..	2	4	1	6	..	3	6	..	..
4 weeks	..	..	..	1	0	2	11	2	1	12	1	..	..	..
2 months	..	..	..	1	4	3	4	2	3	6	12	16	7	..
3 months	..	..	..	1	2	0	6	4	..	6	9	16	3	5
4 months	..	..	..	2	5	1	2	2	1	3	6	8	4	..
5–6 months	..	..	2	1	4	1	..	..	..	..	..	..	1	..
6–7 months	..	..	1	1	..	..	..	..	..	..	..	..	..	..
8 months	..	..	1	1	..	..	..	..	..	..	..	..	..	1†
11 months	..	..	..	1	..	..	..	..	..	..	..	..	..	..
12 months	..	..	1	1	..	.	..	..	..	..	..	2†	..	..

* Expressed as 50 per cent or greater agglutination or lysis; ** 0 = no agglutination or lysis; † expressed as 25 per cent agglutination or lysis.

Leptospiremia was present in 10 of the 13 pigs inoculated subcutaneously with the bovine strain, while positive blood cultures were obtained from 2 on day 7, 3 on day 8, 3 on days 7 and 8, and from 2 on days 7 through 9.

The 4 hogs inoculated with the porcine strain had leptospiremia of three to ten days' duration (table 1); the greatest number of leptospiras was present in the blood on day 7. Serum antibody was detectable as early as day 7 in 2 hogs; the levels were 10^{-1} and were barely detectable (25% agglutination). The agglutination reactions during the end of the leptospiremic phase also were weak (only 25% agglutination), but these reactions were distinguishable at 10^{-4} at this time. By day 10, all four serums were producing strong agglutination reactions of 50 per cent agglutination or more at dilutions of 10^{-2} or 10^{-3}. After marked agglutination occurred, the blood cultures did not contain leptospiras (table 1).

Antibodies for *L. pomona* were detected in dilutions of 10^{-2} or 10^{-3} by day 8. Maximum antibody response (10^{-7} to 10^{-8}) usually occurred during week 3 to 4, but diminished considerably (10^{-2} to 10^{-5}) by postexposure month 4 or 5. Titers persisted for as long as one year (tables 2, 3).

Urinary antibody levels increased more gradually to a maximum titer of 10^{-3} than did serum antibody levels. The highest levels were observed at three or four months and appeared to be independent of the serum titer. Urine titers were demonstrable, although weak, for one year (table 2).

The maximum number of leptospiras was excreted in the urine during days 20 to 30 (tables 3, 4, 5) and were readily

TABLE 3—Correlation of Urinary pH, Urinary Antibody, Serum Antibody, and Leptospiruria for 11 Hogs

Hog	Months after exposure	Urine		Leptospiruria		Serum titer*
		pH	Titer*	Dark-field	Animal inoculation	
4921	3	6.25	10^{-2}	—	—	10^{-6}
4922	3	6.6	10^{-1}	—	+	10^{-6}
4958†	3	6.9	10^{-1}	—	+	10^{-7}
4960	3	5.45	10^{-2}	—	—	10^{-7}
4961	3	6.55	0	....	+	10^{-7}
4961	3.5	6.85	0.5×10^{-1}	—	+	10^{-7}
4962	3	6.3	0.5×10^{-1}	—	+	10^{-5}
4963	3	5.75	0	—	—	10^{-5}
4970	3	6.75	0.5×10^{-1}	—	+	10^{-7}
4971	3	6.55	0.5×10^{-1}	—	+	10^{-6}
4971	3.5	5.9	10^{-2}	....	—	10^{-3}
4972	3	6.3	0.5×10^{-1}	....	—	10^{-6}
4921	4	6.05	10^{-1}	—	—	10^{-4}
4958†	4	5.5	10^{-2}	—	—	10^{-3}
4963	4	6.75	10^{-1}	—	+	10^{-4}
4969	4	5.1	0.5×10^{-1}	—	—	10^{-4}

* Titers expressed as 50 per cent or greater agglutination or lysis; † hog 4958 was infected by contact with infected pigs. Other pigs were infected with the bovine strain after subcutaneous inoculation.

TABLE 4—Duration of Leptospiruria and Persistence of the Porcine Strain in Hog Kidneys After Exposure at 7 Months of Age

Hog	Urine samples* Weeks 1–2	2–3	3–4	4–5	7–8	8–13	Last day of leptospiruria	Necropsy examination: Kidneys (bacteriological)	Necropsy examination: Serum titer**
4927	0/1†	.....	.....	0/6	0/2	1/9	70	Negative at 102 days	10^{-3}
4973	0/2	4/6	.....	3/3	0/2	0/6	32	Negative at 146 days	10^{-4}
4928	1/5	2/3	3/3	2/2	.....	1/13	61	Was not killed	.....
4929	1/4	3/5	1/1	3/3	.....	0/12	31	Was not killed	.....

* Uncentrifuged samples were examined by dark field (x 590) and, if positive, animal inoculations were not made. All dark-field negative samples were inoculated into guinea pigs to ascertain presence of leptospiras. † Numerator denotes number of positive samples, while denominator represents total samples examined. ** 50 per cent or greater agglutination or lysis end point.

demonstrable in uncentrifuged urine by dark-field microscopy at x 590. Leptospiras were so numerous that the field under examination resembled one obtained from a culture. Three months postexposure, leptospiras were not demonstrable by dark-field examination, but the same urine samples were positive by animal inoculation techniques (table 3). Leptospiruria lasted 122 days in 1 animal. The kidneys of this animal contained no leptospiras at 151 days. Kidneys from 2 of 6 swine killed at 121 days contained leptospiras. This time, approximately 122 days, represents the maximum duration of renal leptospiral residence in these experimentally infected swine (table 5).

Leptospiras were not found in urine which had antibody levels higher than 10^{-1}, but often were present when serum titers were over 10^{-5}. Not all urine from swine with high serum reactions yielded leptospiras. Only 1 animal which had a serum titer of 10^{-4} was excreting the organism (tables 3, 4). The leptospiras were not observed in urine with a reaction lower than pH 6.3 (table 3).

The differences between the average daily gain of *L. pomona*-infected swine and that of their uninfected litter mates was not statistically significant (table 6). One infected pig (4972) did not make satisfactory gains but, at necropsy, a chronic, low-grade pneumonia and ascariasis were evident. These conditions alone would account for the meager gains made by this animal.

DISCUSSION

The course and signs of leptospirosis in feeder-type pigs are often unapparent, and consist of mild pyrexia, transient anorexia,

TABLE 5—Duration of Leptospiruria and Persistence of the Bovine Strain of Leptospira Pomona in Porcine Kidneys

Hog	Urine samples* Months 1	2	3	4	Last day of leptospiruria	Necropsy examination: Bacteriological examination of kidneys	Necropsy examination: Serum titer‡
4968†	2/2**	.....	.....	.....	34	Positive at 35 days	10^{-8}
4974†	1/1	.....	.....	.....	29	Positive at 49 days	10^{-6}
4967	1/1	1/1	.....	.....	55	Positive at 76 days	10^{-8}
4960	.....	1/2	0/3	.....	63	Positive at 121 days	10^{-7}
4959	.....	.....	0/1	.....	....	Positive at 121 days	10^{-6}
4922	.....	0/3	1/3	.....	93	Negative at 121 days	10^{-7}
4961	1/1	1/3	2/4	.....	101	Negative at 121 days	10^{-6}
4962	.....	2/2	1/2	.....	93	Negative at 121 days	10^{-5}
4970	2/2	0/2	1/3	.....	93	Negative at 121 days	10^{-4}
4921	.....	1/2	0/2	0/1	34	Negative at 151 days	10^{-3}
4963	1/1	0/1	0/2	1/2	122	Negative at 151 days	10^{-2}
4971	2/2	2/3	1/2	.....	101	Negative at 184 days	10^{-2}
4972	.....	3/3	0/1	0/1	44	Negative at 214 days	10^{-2}
4923	1/1	0/1	0/1	0/2	30	Was not killed	.....
4969	1/1	0/1	0/1	0/2	29	Was not killed	.....
4958†	3/3	1/2	0/1	.....	53	Was not killed	.....

* Composite results represent approximately 50 per cent obtained by dark-field examinations (x 590) of uncentrifuged urine and remainder by animal inoculation procedures; † infected at 4 months of age by contact with infected hogs. Other hogs were infected subcutaneously when 3 months old. ** Numerator denotes number of samples positive, while denominator indicates total samples examined; ‡ 50 per cent or greater agglutination or lysis end point.

TABLE 6—Weight Gain and Feed Consumption of Leptospira Pomona-Infected Swine in 115 Days, as Compared with Litter Mate Controls

Item	Control pigs		Infected pigs	
	Pen 1	Pen 2	Pen 3	Pen 4
No. pigs	5	5	5	5
Average initial weight (lb.)	34.6	26.3	34.9	25.7
Average final weight (lb.)	164.6	171.0	189.6	149.4*
Average daily gain (lb.) ± s.e.	1.12 ± 0.06	1.26 ± 0.11	1.35 ± 0.08	1.08 ± 0.22
Average daily feed (lb.)	3.86	4.41	4.29	3.57
Pounds of feed per pounds of gain	3.44	3.51	3.19	3.31

* At 115 days, 1 pig in this lot weighed 68 lb., which was 50 per cent less than any other pig being tested.

and moderate malaise. On the other hand, severe manifestations have been reported in pregnant sows and their young in the naturally occuring disease. These include hemoglobinuria, icterus, anemia, and convulsions, as well as abortion, abnormal milk, and reduced milk flow.[3, 8, 10, 24] Experimental infections in feeder-type swine do not appear to present as severe a syndrome as described for field cases, *i.e.*, the first four manifestations mentioned. Young swine have been shown to be susceptible to *L. pomona* either by subcutaneous inoculation or by pen contact exposure. Pregnancy, concurrent parasitism, and bacterial or viral infections, as well as nutritional deficiencies, undoubtedly aggravate the course of *L pomona* infection.[11] In this respect, the severity of leptospirosis may be influenced by the stresses to which the host is subjected.

Apparently, the greatest losses due to leptospirosis are attributable to abortions and poor weight gains in pigs born to recently infected sows. Feeder swine, infected at 10 to 12 weeks of age or older, make satisfactory gains (table 6). From a practical standpoint, it is a mistake to recommend that young pigs, raised under conditions of good husbandry, be sent to slaughter before they reach suitable market weight because of *L. pomona* infection per se.

Swine are prime reservoirs of leptospirosis *(L. pomona)* for both animals and man, for several reasons. First, a prolonged period of leptospiremia occurs, during which considerable numbers of leptospiras are present in the blood of essentially asymptomatic swine. Slaughter of such animals exposes abattoir personnel; and uncooked meat scraps, if consumed, might infect other swine. Second, porcine urine at 20 to 30 days after infection contains considerable and, at times, maximum numbers of leptospiras, and contamination of feed, bedding, and water occurs readily, with consequent spread of infection. Third, hogs excrete leptospiras in their urine longer than do other farm animals.

Leptospiras were cultured from the blood of swine when serum antibody was detected at dilution levels of 10^{-2} to 10^{-4}. Apparently, leptospiremia is not terminated absolutely with the appearance of the specific antibody. Antibody often is first detected at serum dilutions of 10^{-3} or 10^{-4}, and remains at these levels for two to three days. For example, on day 8, the serum of hog 4928 (table 1) produced 50 per cent or more agglutination at dilutions of 10^{-1} and 10^{-2}, while 25 per cent agglutination was seen at dilutions of 10^{-3} and 10^{-4}. On day 9, none of the serum dilutions produced 50 per cent agglutination, yet dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} gave 25 per cent agglutination. It is theorized that *in vivo* lysis of large numbers of leptospiras causes neutralization of circulating antibody. The drop in titer observed in 3 of the 4 hogs on day 9 (table 1) thus may be explained. One hog (4973) had a reduced hemoglobin level and erythrocyte count on day 8, while 2 had slight anisocytosis and reticulocytosis at nine to 13 days postexposure. It can be postulated that lysis of leptospiras in the blood by antibody (1) neutralizes antibody and lowers the serum titer, and (2) releases a hemolytic endotoxin from the leptospiral cell or forms a hemolysin complex with specific antibody. The latter leads to erythrocyte destruction with consequent anisocytosis, reticulocytosis and, in severe cases, hemoglobinuria. This same sequence of events within comparable time intervals has been reported in lambs experimentally infected with *L. pomona*.[19] Only in the most severe cases will such signs be observed.

The excretion of viable leptospiras in

urine is dependent upon the pH and presence of leptospiral antibody in the urine. A medium of pH 6.0 or lower is unfavorable for survival of the microorganisms.[21] The ration fed will, to some extent, influence urinary pH. Leptospiral antibody in the urine will tend to remove the leptospiras and will prevent their survival by immobilization and eventual lysis. The organisms generally are not observed in urine at pH 6.0 or lower when the antibody level is 10^{-2} or higher (table 2). However, leptospiras can be present in blood with a titer of 10^{-2} (table 1); possibly, the blood proteins act as protective colloids. These factors, as well as others, may account for the persistence of the renal carrier (shedder) state in swine and explain the fact that the duration of the carrier state was observed for one year[22] but that only 122 days appeared to be maximal during this investigation.

Direct dark-field microscopic examination of urine is not considered a reliable technique for ascertaining leptospiruria. Leptospiras are often present but can not be observed by direct examination, yet enough are present to infect guinea pigs or hamsters when introduced intraperitoneally. Bauer[1] found that as few as ten cells of either the bovine or porcine strain will infect either laboratory animal. Neither sheep[13,19] nor dogs[7] shed sufficient numbers of leptospiras in the urine to be observed by dark-field microscopy, yet the presence of the microorganisms is readily demonstrable by animal inoculation techniques.

Leptospiral antibody in the urine probably is not a result of blood or globulin leakage, but a product of reticuloendothelial cells within the kidney. The basis for this assumption is that kidney damage is greatest two to four weeks postinfection; the highest serum antibody titers are observed at three weeks, whereas maximum urinary antibody titers occur at about three months (table 2). Similar observations have been made in pregnant ewes.[13]

Significant differences between the virulence of the porcine and bovine strains of *L. pomona* were not discernible. Both strains had been kept in continual guinea pig passage since isolation from field cases. A portion of the same inoculum of the bovine strain which infected the swine proved to be highly virulent for lambs.[19] Guinea pig serial passages of the porcine strain two months before use in these investigations and eight months later proved to be virulent for lambs[19] and dogs.[7] The bovine strain has proved capable of infecting cattle,[17,20] goats,[16,20] and sheep,[13,19] and dogs.[7] Transmission from cattle to swine and goats,[20] as well as from swine to sheep and goats,[19] during pen contact was easily established, using the bovine strain. These facts would indicate that both the bovine and porcine strains used in the investigations were comparable in virulence to field strains of *L. pomona*.

Serological titers for *L. pomona* have been observed to persist in cattle for as long as 52 months.[15] The serum antibody level in the experimentally infected swine was evident at 10^{-2} and 10^{-3} one year after exposure. Only a remote possibility exists that the swine were infected at that time. Urinary antibody was demonstrable at one year; however, the agglutination reaction was weak. It is the opinion of the authors that serum agglutination-lysis titers of 10^{-4} or higher, as determined in this laboratory, probably indicate active infection, a renal carrier state, and considerable potential hazard to livestock and human health. Serum antibody detectable at dilutions of 10^{-2} to 10^{-3} indicate previous infection or an early active infection of one to two weeks' duration. Interpretation of serological titers is, therefore, best made in close collaboration with the clinician.

SUMMARY

Twenty swine were infected with *Leptospira pomona*, 17 by subcutaneous inoculation and 3 by contact with infected swine. Sixteen were infected with a strain of Leptospira isolated from cattle, while 4 were infected with a strain obtained from swine.

The only signs of infection were a mild elevation in temperature, a transient anorexia, and an almost imperceptible malaise in a few animals.

The blood cell values, hemoglobin levels, and blood nonprotein nitrogen values remained essentially normal for most animals throughout the investigation, with the exceptions of slight anisocytosis and reticulocytosis in 2 swine, and a decrease in both erythrocytes and hemoglobin in 1 animal.

Leptospiremia was observed for as long as ten days after inoculation. In three instances, leptospiras were present in the blood at the time when serum antibody

was demonstrable. Serum antibody was still present one year after exposure, although the highest titers were observed at three weeks.

The maximum number of leptospiras were excreted in the urine 20 to 30 days after exposure. The leptospiruria persisted in at least 2 swine for 122 days. The various factors influencing leptospiruria, e.g., urine pH and urinary *L. pomona* antibodies, were investigated. Urinary antibody was demonstrable after one year, but only weak reactions were observed. Highest titers were seen approximately three months after exposure.

Theories regarding leptospiral hemolytic anemia and production of urinary leptospiral antibody are discussed.

Weight gains of 10 infected swine were comparable to those observed for 10 uninfected litter mate controls.

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Experimental Leptospirosis. V. Pathology of *Leptospira Pomona* Infection in Swine

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IT HAS OFTEN been said of research that experiments on any given project should be repeated several times at different laboratories in order to learn the true manifestations of a disease. The pathological changes produced in swine by infecting them with *Leptospira pomona* have been reported only once previously, by Burnstein and Baker.[1] In view of the paucity of reports, it was deemed advisable to learn more about *L. pomona* infections in swine and to report the findings.

MATERIALS AND METHODS

Eighteen swine were used in this experiment, 3 control and 15 infected animals. The infection was produced by inoculating 11 pigs subcutaneously with a bovine strain of *L. pomona* and 2 with a porcine strain. The other 2 pigs developed the disease by direct contact with other infected pigs. Additional information on the history, clinical data, and bacteriology have been presented in the preceding paper of this series.[5]

The 2 pigs that developed leptospirosis from contact were killed 35 and 49 days after transmission had occurred. The remaining swine were killed 76, 102, 121 (6 pigs), 146, 151 (2 pigs), 184, and 214 days after inoculation with *L. pomona*.

Tissues were saved from the kidneys, livers, spleens, renal lymph nodes, hearts, adrenal glands, and thyroid glands. The brains and spinal cords were obtained from only 3 swine. Immediately after necropsy, all tissues were placed in one or more of the following fixatives: 10 per cent formal-saline solution, Zenker's fluid, and Carnoy's fluid.

The following staining procedures were used: hematoxylin and eosin, Verhoeff's stain for elastic tissues, Heidenhain's aniline blue, periodic acid Schiff (PAS), Sudan IV for fat, Bauer-Feulgen reaction for glycogen, and Levaditi's method for spirochetes.

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TABLE 1—Pathological Changes in Kidneys of Leptospira Pomona-Infected Swine

	Course of infection*														
	35†	49†	76	102	121	121	121	121	121	121	146	151	151	184	214
Infiltration of lymphocytes, plasma cells, and macrophages	+++	+	+++	−	+	+	+	+	+	+	++	++	++	++	−
Necrosis of distal convoluted tubules	−	−	−	−	+	+	++	−	++	−	+	−	+	−	−
Increase of connective tissue	+	−	++	−	+	+	+	+	+	+	+	−	+	++	−
Inflammation of major and minor calyces	++	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Presence of fat in tubular epithelium	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−
Atrophy and necrosis in inflamed areas	+++	+	+++	−	+	+	+	+	−	−	++	+	+	++	−
Presence of protein in subcapsular spaces of renal corpuscles	−	−	−	−	−	−	−	++	−	++	+	−	+	−	−
Areas of fibrosis and hyalinization	−	−	−	+	−	−	−	−	+	−	−	−	−	+	++
Hyalinized thickening of the glomerular tufts	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−
Increase of goblet cells in transitional epithelium	−	−	++	−	−	−	−	−	−	−	−	−	−	+	−
Silver stain for Leptospira	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−

* Time elapsing between exposure and death; † time represents approximate day of transmission to death. +++ = extensive; ++ = moderate; + = slight; − = negative.

RESULTS

The macroscopic changes were confined to the kidneys and renal lymph nodes. The capsules of the kidneys stripped easily from the cortices, except in a few minor instances. The cortices had scattered to numerous grayish white foci, ranging in size from the limit of visibility up to 1 cm. in diameter (fig. 1, 2). Some of the lesions were raised slightly above the surface, while a few were somewhat depressed in the center. When the kidneys were sectioned sagittally, some of the grayish white foci extended through the cortex into the medulla. The renal lymph nodes were slightly enlarged and edematous.

Microscopically, the kidneys showed varying degrees of inflammation which involved all parts. All the infected pigs, except those killed 102 and 214 days after inoculation, had an intertubular and perivascular infiltration with lymphocytes, plasma cells, and some macrophages (fig. 3, 4, 5). A few of the inflammatory cells were undergoing mitosis. Segments of the nephrons in the inflammatory areas were atrophic and necrotic and, in many instances, had completely disappeared. Of the swine necropsied 121 days or more after inoculation, 6 showed pyknosis of the epithelial cells of the distal convoluted tubules (fig. 6). The medullary papillae (fig. 7) and calyces were often infiltrated with a cellular exudate. All the swine except 4 had active proliferation of fibroblasts and the formation of collagenous fibers (fig. 8). This was observed primarily between the tubules of both cortex and medulla. The increase of connective tissue was not extensive in any of the swine. Pigs necropsied 102 and 214 days postexposure had no active areas of inflammation, but did have areas of replacement fibrosis and hyalinization.

The renal corpuscles had only a few minor changes. In several kidneys, Bowman's capsule was surrounded by inflammatory cells. In 4 swine, the subcapsular spaces contained some proteinaceous material. In 2 pigs, there was some hyalinization of the glomerular tufts. One of the pigs necropsied 121 days postexposure showed a peculiar pathological change; some of the glomeruli had undergone disintegration and lysis, until all that remained were erythrocytes and cellular debris (fig. 9).

The pigs necropsied 76 and 184 days postexposure had thickened and vacuolated transitional epithelium (fig. 10) which gave a PAS reaction characteristic of mucin.

Silver-stained sections of the kidneys from pigs necropsied at 35, 46, and 76 days were examined for *L. pomona*. A few or-

Fig. 1—Kidneys of 2 pigs killed 151 days after exposure to Leptospira pomona, showing grayish white foci in the cortices. x ½.

Fig. 2—Kidney of a pig necropsied 35 days after exposure, showing grayish white foci in cortex. x ⅝.

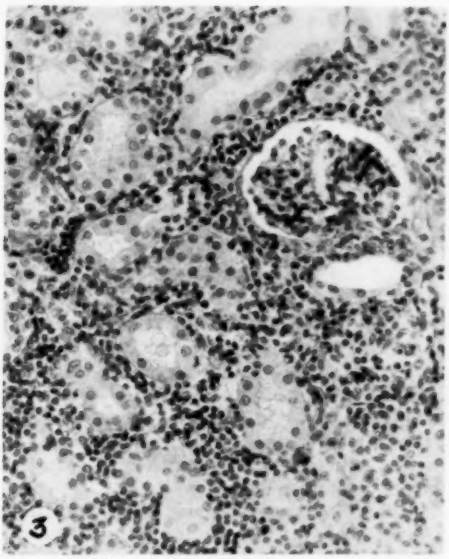


Fig. 3—Section of the renal cortex of a pig necropsied 76 days postexposure, showing the infiltration of lymphocytes, plasma cells, and some macrophages in the intertubular areas. x 200.

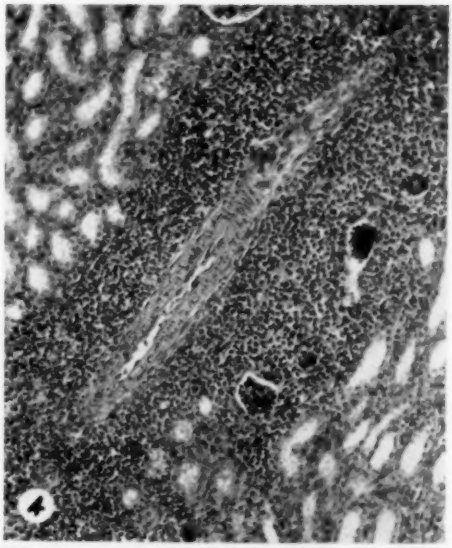


Fig. 4—Section of the kidney of a pig necropsied 76 days postexposure, showing the perivascular areas. Notice the extensive infiltration of cells, similar to that shown in figure 3. There are heterophil infiltrations in some of the tubules. x 92.

ganisms were found in the lumina of the proximal convoluted tubules of the pig necropsied 35 days postexposure. *Leptospira pomona* was isolated at necropsy from these 3 swine. A summary of the kidney lesions is shown (table 1).

The renal lymph nodes in the pigs necropsied 76 and 146 days postexposure had extensive edema. In these areas, the lymphocytes were separated by the fluid, giving a sparse appearance (fig. 11).

The livers were normal except that in 1 of the pigs necropsied 121 days postexposure, which had some pyknosis of the hepatic cells at the center of the lobule. The cytoplasm of the hepatic cells in all the swine was foamy in appearance, due to the presence of glycogen (Bauer-Feulgen reaction).

No pathological changes were found in the brains or spinal cords of the 3 swine examined.

DISCUSSION

The two most important macroscopic changes in the *L. pomona*-infected pigs were scattered to numerous grayish white foci in the kidneys and edema of the renal

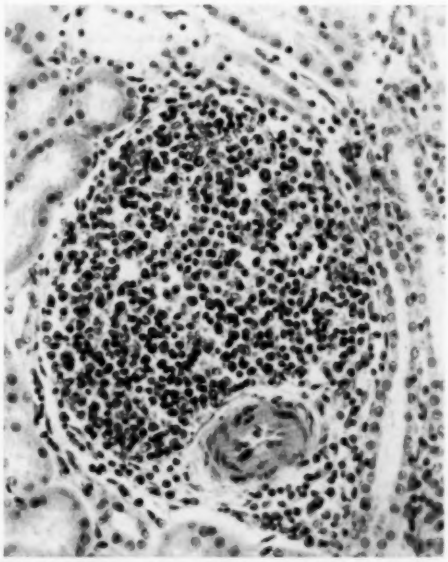


Fig. 5—Section of a kidney of a pig necropsied 121 days postexposure, showing focal area of infiltrating lymphocytes, plasma cells, and some macrophages in the glomerulus. x 200.

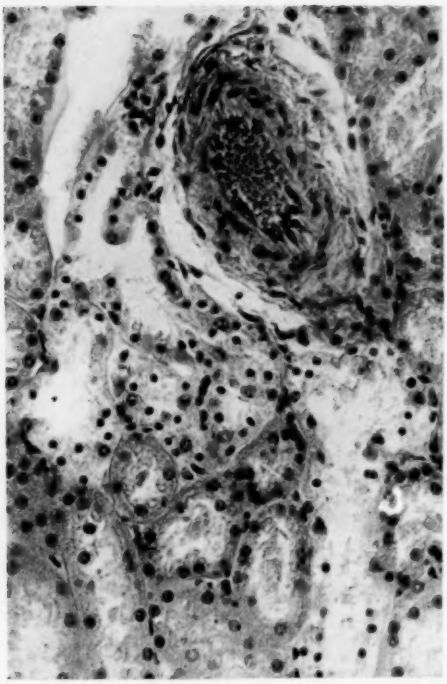
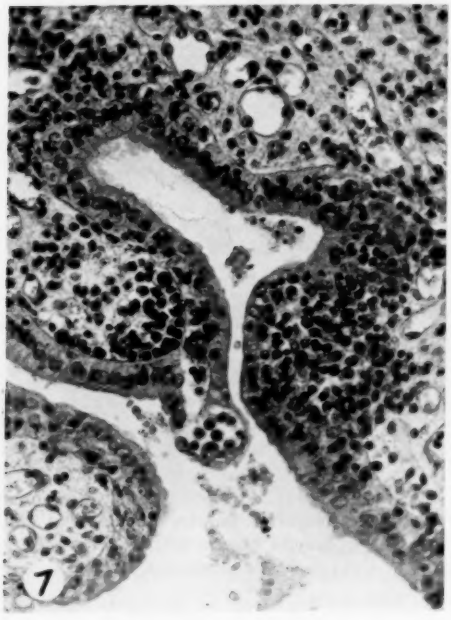


Fig. 6—Section of a kidney of a pig necropsied 121 days postexposure, showing marked pyknosis of the distal convoluted tubules. x 250.



lymph nodes. Icterus was not a feature; yet, in swine infected with *Leptospira icterohaemorrhagiae*, it was a constant finding.[2,5] Gochenour *et al.*[3] reported petechial hemorrhages in the visceral organs and intestinal mucosa, which we did not find. However, some of their cases were complicated by *Salmonella choleraesuis* infection. Burnstein and Baker [1] observed hemorrhages in the lungs of 3 pigs.

Microscopically, the changes in the kidneys of our experimental pigs were similar to those reported by Burnstein and Baker,[1] with a few minor exceptions. Our pigs apparently had more degenerative changes in the nephrons. In 7 of their pigs, there were extensive changes in the liver, characterized by pyknosis, karyorrhexis, and granularity of the cytoplasm. Only 1 pig in this experiment had significant pyknosis of the hepatic cells.

Inflammatory processes in the kidneys appear to continue for long periods after

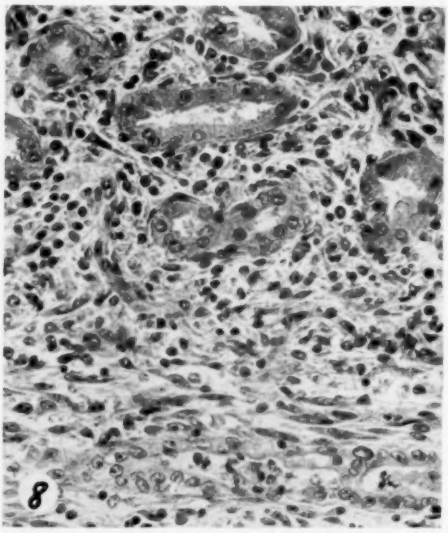


Fig. 8—Renal cortex of a pig necropsied 76 days postexposure, showing marked proliferation of fibroblasts and the formation of connective tissue. Notice the atrophy and disappearance of tubules.

x 250.

←

Fig. 7—Papilla of kidney of a pig necropsied 76 days postexposure, showing infiltration of lymphocytes and plasma cells even in the transitional epithelium. x 250.

L. pomona can no longer be demonstrated in the urine. In this study, leptospiras were not found after 121 days; yet, active inflammation was still present in the pig necropsied 184 days postexposure.

Proliferation of connective tissue and fibrosis began to develop in the kidneys of swine about 35 days postexposure. This was not true in experimental ovine infections.[4] In 8 sheep, little connective tissue was evident after 105 days.

Leptospira pomona was difficult to find in the kidneys using special silver stains. Field and Sellers[2] and Nisbet[6] were able to demonstrate *L. icterohaemorrhagiae* readily in livers and kidneys, using Levaditi's stain.

SUMMARY

1) The effects of *Leptospira pomona* infections were studied in 15 swine.

2) *Leptospira pomona* infections can lead to widespread renal damage.

3) Grossly, the kidneys showed scattered to numerous grayish white foci in both the cortex and medulla.

4) Microscopically, these grayish white foci consisted of numerous lymphocytes, plasma cells, and some macrophages. Some of the lesions had an increase of fibroblasts and collagenous fibers.

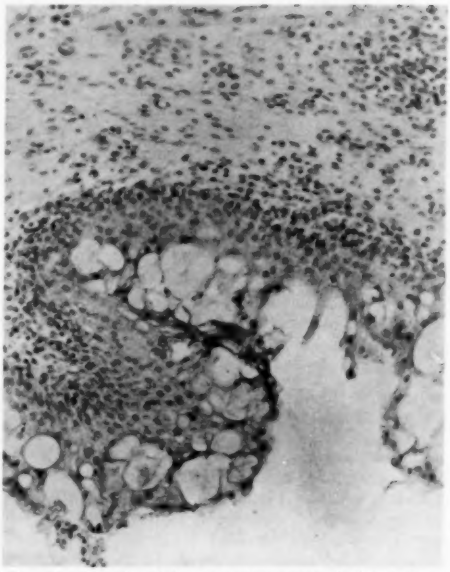


Fig. 10—Renal pelvis of a pig necropsied 184 days postexposure. Notice the vacuolated condition of transitional epithelium; special stains (periodic acid Schiff) indicate mucus. x 115.

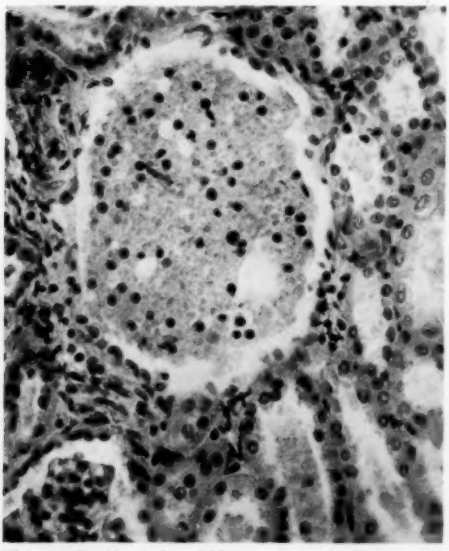


Fig. 9—Section of a kidney of a pig necropsied 212 days postexposure, showing a glomerulus that is undergoing autolysis. x 250.

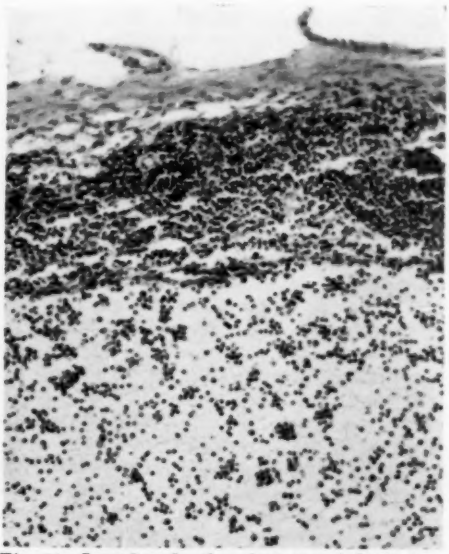


Fig. 11—Lymph node of a pig necropsied 146 days postexposure, showing edema. x 115.

5) The proximal and distal convoluted tubules showed varying degrees of degeneration and necrosis.

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The Influence of Diet on the Development of *Ascaris Suum* in the Small Intestines of Pigs

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IT HAS BEEN KNOWN for 40 years that *Ascaris suum* of pigs has a direct life cycle.[4,5] However, adult worms do not always result when infective Ascaris eggs are fed.[3-6,8-10]

During our investigations over the past three years, large doses of infective *A. suum* eggs were fed to 19 pigs which were examined at least 22 days later. No worms were found in 8 pigs and the remaining 11 harbored an average of 23 worms each.[2] For this reason, experiments were begun for the purpose of devising effective methods of establishing ascarids in the small intestines of pigs. In the experiments reported here, the pigs were either starved to slow the intestinal motion, or were placed on diets consisting solely of rolled oats.

MATERIALS AND METHODS

All of the pigs were obtained by hysterectomy, deprived of sow's colostrum, and raised in strict isolation to prevent uncontrolled infection with *A. suum*.[12]

Ascaris suum eggs were taken from the uteri of worms which had been acquired from an abattoir. They were mixed with granular bone charcoal, incubated for six to eight weeks at 27 C., and then maintained at 5 C. until used. The eggs were proved infective before each experiment by producing pneumonia when fed to mice.

Dilution methods were used to determine the approximate number of eggs given to each pig. Several 1-Gm. samples of the culture were weighed into egg-counting jars, and eggs counts were made by the Stoll dilution method.[11] After the number of eggs per gram was determined, a quantity sufficient to yield the desired dose was weighed into a flask, water was added, and the eggs were separated from the charcoal by shaking. Charcoal particles were then removed by pouring the mixture through a soil sieve. The eggs were concentrated into the proper amount of water by centrifugation and were given to the pigs by stomach tube.

The number of larvae migrating in the liver and lungs of the pigs was estimated at necropsy by counting the number present in each of four samples (0.5 Gm.) of these tissues, and multiplying the average number of larvae per gram by the total weight of the organs. The number of larvae recovered from the trachea and gastrointestinal tract was added to the number of larvae from the liver and lungs to obtain the total number present.

EXPERIMENTS AND RESULTS

Experiment 1.—An attempt was made to establish adult ascarids by starving the pigs to slow the intestinal emptying time.

Six Yorkshire pigs, 18 days old, were given infective *A. suum* eggs. Of the 6 pigs, 3 (principals) were given one half their usual ration (1/12 rather than 1/6 pt. of milk twice daily) on the previous day, and no morning feed on the day the eggs were administered. Each was given approximately 30,000 infective Ascaris eggs. Each of the remaining 3 pigs (controls) was also given 30,000 eggs at this time, but received their regular ration (1/6 pt. of milk) prior to and after the eggs were administered. The 3 principals were given only one half of their evening ration that day. On postinfection day 7, the ration of the principals was again reduced to one half, and on days 8 and 9 they were given only water, in the same quantity as their former ration of milk (1/12 pt.). The water was given in four daily portions. On postinfection day 10, one half ration of milk (1/12 pt.) was substituted for the water, and after day 10, they were again given their regular ration (1/6 pt. of milk twice daily).

All 6 pigs were observed for signs of pulmonary larva migration from postinfection days 6 to 15, and daily egg counts were made from postinfection day 54 until the pigs were killed and examined (day 86).

Results.—Signs of respiratory dysfunction appeared in 1 pig on postinfection day 6 and persisted until day 11. Of the remaining pigs, 1 was severely affected, and 4 displayed only mild signs until postinfection day 12. There was no difference in the severity of the signs of respiratory dysfunction in the differently treated animals. No eggs were found in the feces, and no ascarids were found in the intestinal tracts of any of the pigs at necropsy on day 86.

Experiment 2.—The influence of a diet of rolled oats on establishing ascarids was observed.

Four Hampshire pigs were maintained on a diet of fortified milk until they were 7 days old, when

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the milk was replaced by rolled oats. Upon reaching the age of 8 days, each pig was given 100,000 Ascaris eggs.

Results.—Of the 4 pigs, 2 died from ascariasis on postinfection day 9. Totals of 36,000 and 56,000 larvae, respectively, were estimated to be migrating in these pigs. A third pig stopped eating, weakened, and died on postinfection day 22. It is suspected that the 657 subadult ascarids which were recovered from the intestine of this pig were instrumental in causing its death, since no lesions of disease were found. The fourth pig continued in apparent good health and, when it was killed on postinfection day 35, 164 ascarids were present in the small intestine.

Experiment 3.—Three Hampshire pigs were placed on a diet of rolled oats when they were 14 days old and, three days later, each pig was given 100,000 infective Ascaris eggs.

Results.—All 3 pigs displayed signs of pulmonary larva migration. Two of these pigs died on postinfection day 11, possibly because of the effects of the migrating larvae, since 18,000 larvae were found in 1 pig and 29,000 in the other. The third pig was killed on postinfection day 46. It was 63 days old, weighed only 6 lb., and 59 worms were present in its small intestine.

Experiment 4.—Each of 4 pigs, 60 to 65 days old, was given 100,000 infective Ascaris eggs. Simultaneously, they were placed on a diet of rolled oats. All of the pigs were killed and examined on postinfection day 50.

Results.—Signs of pulmonary larva migration were apparent in all of these pigs. At necropsy, 120, 115, 14, and 9 worms, respectively, were found in the intestinal tracts of the 4 pigs.

Experiment 5.—The number of worms developing when pigs on a diet of oats were given a single dose or divided doses of Ascaris eggs was compared.

Eight pigs, 7 Yorkshires (7 weeks old) and 1 Duroc-Jersey (5 weeks old), were used. Of the Yorkshires, 4 were given a single dose of 100,000 eggs. The remaining 3 Yorkshires and the 1 Duroc-Jersey were each given four doses of 25,000 eggs at three-day intervals.

Results.—Signs of pulmonary larva migation were apparent in all of the pigs from postinfection day 7 to day 14. Two of the pigs which were given a single dose of 100,000 eggs died from pulmonary larva migration. One died on postinfection day 7, and 23,000 larvae were estimated to be present in the lungs and trachea. The other pig died on day 10, and 13,000 larvae were found in the lungs, stomach, and small intestine.

Feces from the remaining 5 pigs were examined on postinfection day 60, and Ascaris eggs were found in all but 1 pig. At necropsy, 302 immature worms were found in the intestinal tract of this pig.

Ascarids were found in the intestines of all of the pigs at necropsy on postinfection day 61. The 2 pigs which were given single doses of 100,000 eggs each harbored 111 and 22 worms, respectively. The 4 pigs which were given four doses of 25,000 eggs each harbored 395, 302, 192, and 5 worms, respectively.

Experiment 6.—The number of ascarids which resulted when a small initial dose of Ascaris eggs was followed by a larger dose 30 days later was estimated.

Three Hampshire pigs, 9 weeks old, were each given 10,000 eggs in two doses of 5,000 eggs, three days apart. Of the 3 pigs, 2 were given 50,000 eggs in a single dose 29 days later. At the same time, a fourth pig was given its initial dose of 50,000 eggs. The pigs were killed and examined for worms 75 days after being given the first dose, and 44 days after the last dose of eggs.

Results.—The 3 pigs which were given the initial doses of 10,000 eggs, showed signs of pulmonary larva migration from postinfection day 11 to day 13. At necropsy, the 2 pigs which were given the additional 50,000 eggs harbored 128 and 43 worms, respectively, while the pig which was given only 10,000 eggs had 42 worms. No worms were found, and there were no signs of respiratory dysfunction, in the pig which was given only the 50,000-egg dose at 13 weeks of age.

Feces of all pigs were first checked 67 days after the initial doses and were found to contain Ascaris eggs.

DISCUSSION

In the most complete studies of *A. suum* which have been reported, Ransom and Foster,[4, 5] Martin,[3] and Roberts [6] gave *A. suum* eggs to a total of 31 pigs which were examined at least 22 days later. Only 16 of the 31 pigs harbored ascarids at necropsy. Only vague reference is made by these authors as to the number of eggs given, but the number was assumed to be sizable since, in almost every instance, verminous pneumonia resulted. Infected pigs had an average of 14 worms each, and 9 of them harbored less than 10 worms each. The greatest number present was 73 worms in 1 pig. The diet of the pigs was not clearly defined.

In the five experiments reported here, in which pigs were fed rolled oats, 17 pigs

were given infective *A. suum* eggs and were examined at least 22 days later. All but 1 of these pigs harbored ascarids, the average number being 139 worms, and only 2 of the infected pigs had less than 10 worms each.

Spindler [7] fed 12,000 Ascaris eggs daily to 4 weaned pigs for a total of 18 days. At necropsy, 126 days after the experiment began, they harbored 109, 39, 20, and 12 worms, respectively. No milk was included in the diet of these pigs, which possibly contributed to the number of worms which were able to become established.

Clapham [1] fed 200 Ascaris eggs to 4 pigs (11 weeks old), twice daily for ten days. All became infected, as indicated by the presence of Ascaris eggs in the feces after the usual prepatent period. She concluded that infections become established more readily when repeated small doses of eggs are given. In experiment 5 of this study, the 2 pigs which were given the single dose of 100,000 eggs each harbored 111 and 22 worms, respectively, while the 4 pigs which were given the 4 repeated doses of 25,000 eggs each had 394, 302, 192, and 5 worms, respectively. There is no significant difference between the number of worms in pigs given single doses and in those given repeated doses (Student's t test).

The constituent of rolled oats which is conducive to the development of *A. suum* is not known. There is a possibility that this diet slowed the emptying time of the small intestine, slowing the current through the lumen, thereby permitting the larval worms to retain their position in the intestine. It is also possible that oats contain an essential food element for larval ascarids, permitting them to grow to adult size. Unpublished data from this laboratory [2] indicate that the food requirements of the fourth-stage ascarid larvae are different than the requirements of third-stage larvae. For example, it was found that third-stage larvae maintained *in vitro* grew for a few days, moulted, then grew no more, although they remained alive for 60 days. Similar phenomena have been observed *in vivo* when pigs being fed only milk harbored a few worms (for 20 days) which were much smaller than the worms of the same age found in the intestine of pigs being fed rolled oats.[2] A further possibility is that the diet of rolled oats had no direct effect on the worms but, by reducing the vitality and resistance of the pig host, it enabled the worms to become established more readily and in larger numbers.

Only a fraction of the larvae migrating through the host were able to become established within the intestine. An average of 29,266 larvae each were found in those pigs which were examined during the first 11 days of the infection, whereas an average of 139 worms each were present in the 17 pigs examined after postinfection day 22.

There is an indication that the larval *A. suum* are slowly expelled from the gastrointestinal tracts of milk-fed pigs. Of 19 milk-fed pigs, examined from 22 to 76 days after Ascaris eggs were given, 11 were parasitized with an average of 23 worms each; while of the 13 pigs examined between 39 and 76 days postinfection, only 6 were parasitized, with an average of 4 ascarids each.[2] These observations of diminishing worm numbers, and those on the stunting effect of milk diets on ascarids, indicate that the milk-fed pig is not a suitable host for *A. suum*. A further discussion of the variation in size of Ascaris populations will be presented in a later paper.

The 13-week-old pig which failed to become infected (experiment 6) may indicate age immunity. His 3 litter mates, infected at 9 weeks of age, displayed signs of respiratory complications at 11 to 13 days postinfection. When 2 of them were later given 50,000 eggs each, along with the previously uninfected pig, they showed no evidence of pulmonary larva migration, and no smaller worms were found at necropsy. This observation may indicate that age immunity in pigs becomes established between the ninth and thirteenth weeks of life.

SUMMARY

Five experiments are described in which "colostrum-deprived, disease-free" pigs were given infective *Ascaris suum* eggs while they were on a diet of rolled oats and water. When 17 pigs in these experiments were examined 22 to 75 days postinfection, each harbored an average of 139 worms. The greatest number found was 657 worms, and the smallest was none in 1 pig.

In a sixth experiment, 6 pigs were given Ascaris eggs while they were on a milk diet. Of the 6 pigs, 3 had their food rations severely reduced during the period when

larvae were migrating and coming back into the intestine. Although signs of severe respiratory dysfunction were present from postinfection days 6 to 12, no worms were present in the intestines at necropsy.

The larvae of *A. suum* became established more readily in the intestines of pigs which were on diets of rolled oats than in pigs which were on diets of milk.

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Urinary Estrogens of the Pregnant Sow

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IN THE PIG, substances possessing estrogenic activity are excreted in the urine at two periods during pregnancy. Küst [8] and Struck,[12] using biological assay, found no estrogenic hormone in the urine during the first three weeks of pregnancy. Between the twenty-third and thirty-first days, estrogenic substances were excreted in amounts of the order of 1,000 to 2,000 m.u. (mouse units) per liter of urine. The estrogenic activity then disappeared and was not detected again until the eleventh or twelfth week. Then, estrogenic substances were excreted in increasing amounts until parturition; the maximum excretion was observed in the fifteenth week of pregnancy.

These observations are supported by later investigations based on biological assay (Faiermark and Bigos,[5] Küst and Struck,[9] Faiermark,[4] and Grunsell and Robertson [6]), and on the fluorescent test of Kober [7] (Roth *et al.*[11] and Grunsell and Robertson [6]).

However, as far as the author is aware, no attempt has been made to identify the estrogenic hormone or hormones excreted in the urine of the pregnant sow. Thus, Robinson [10] states that "Oestrogens of unknown identity are excreted in the urine [of the pregnant sow]."

In this paper, observations are reported which indicate that estrone is the only estrogenic hormone excreted in significant quantities in the urine of the sow in late pregnancy.

MATERIALS AND METHODS

Reagents.—The reagents were of the same grades and from the same manufacturers as those used in an earlier investigation.[13]

Urine.—Urine samples were collected from healthy, full-grown sows of Norwegian breed, and stored without preservative in glass flasks at 4 C. until processed, storage time never exceeding 48 hours. The analyses usually were undertaken immediately after collection of the urine.

Hydrolysis.—To a total volume of 500 ml., representing a convenient aliquot of urine, 30 ml. of 11 N hydrochloric acid was added, and the flasks, fitted with reflux condensers, were put in boiling water for exactly one hour, then cooled under tap water.

Extraction, Purification, Methylation, and Chromatography.—Extraction, purification, methylation, and chromatography of the samples were performed according to the method for human urine described by Brown,[2] with the following minor modifications: *(a)* for methylation, the amount of boric acid was increased from 0.9 Gm. to 1.5 Gm.;[13] and *(b)* for chromatography, n-hexane was used instead of petroleum ether, and 4 per cent ethanol in benzene instead of 2.5 per cent ethanol in benzene.[3]

Color Development.—Color development was performed according to Brown,[2] using the Kober reagent as modified by Bauld.[1] All readings were made in a Beckmann model DU spectrophotometer, with photomultiplier, the sensitivity knob of which was kept in position 1 during the readings. Cuvette dimensions were 10 mm.

RESULTS

The chromatographic isolation of a Kober chromogen present in significant quantities in the estrone fraction from pregnant sows' urine is shown (graph 1). Also shown in the same graph is the chromatographic behavior of a proportionate quantity of pure estrone-3-methyl-ether added to urine from a nonpregnant sow. The two substances clearly show the same behavior when submitted to chromatography on alumina.

TABLE 1—Urinary Estrogens in the Pregnant and Nonpregnant Sow

Sow (No.)	Estrogens (μg./liter of urine)		
	Estriol	Estrone	Estradiol*
120 (Nonpregnant)	0.4	0.5	0.0
145 (Nonpregnant)	1.1	1.0	0.5
105 (43 days prepartum)	3.7	27.0	1.9
105 (14 days prepartum)	8.6	95.3	2.0
105 (8 hours prepartum)	17.4	131.0	3.2

* Calculated as estradiol-17β.

The absorption spectrum from 400 to

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600 mμ of a methylated estrone fraction from pregnant sows' urine, is compared with the absorption spectrum of a proportionate quantity of pure estrone-3-methyl-ether (graph 2). The two samples show maximum absorption at the same wave lengths (516 to 518 mμ).

Some preliminary figures are given (table 1) on the quantities of estrogenic hormones excreted in the urine of non-pregnant and pregnant sows. The results are shown as micrograms per liter of urine, owing to the difficulties encountered when trying to collect a 24-hour sample of urine from pigs.

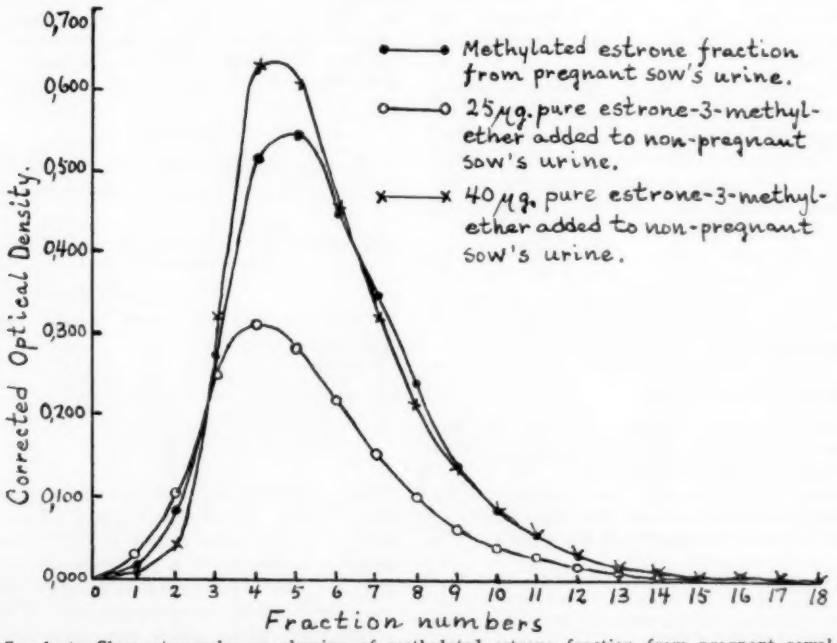
The estrogenic hormones may be excreted in free form, or conjugated to glucuronic, sulfuric, or other acids. Direct ether extractions, performed on unhydrolyzed urine from pregnant sows, showed that only traces of estrone were excreted in free form.

DISCUSSION

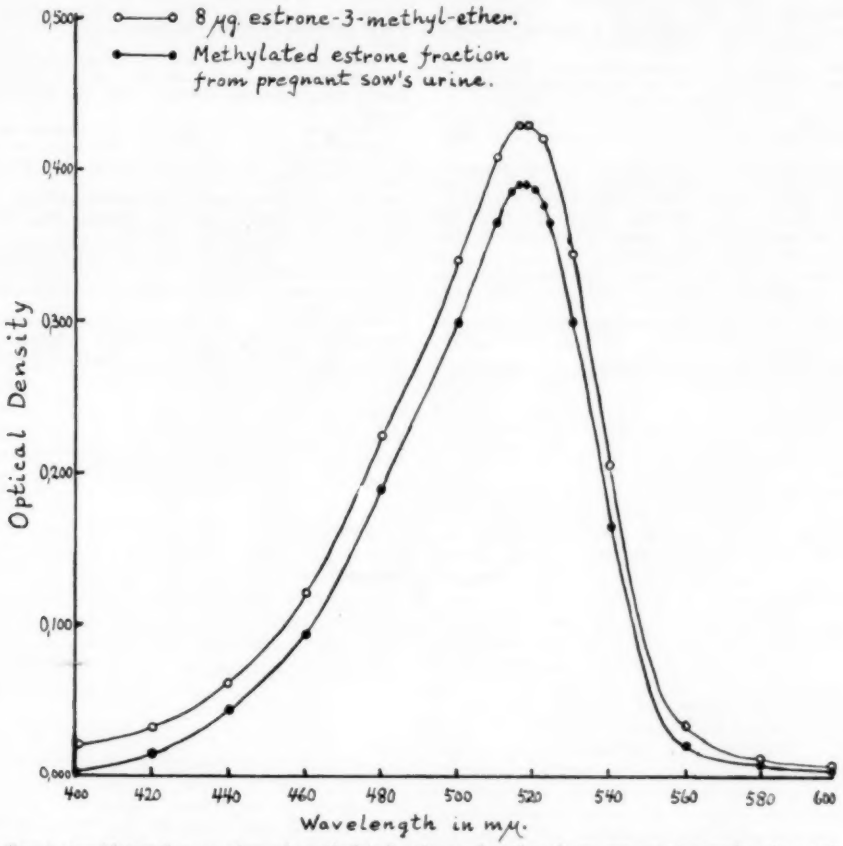
The chromatographic behavior of the methylated estrone fraction isolated from pregnant sows' urine and the conformity between the absorption spectra of the methylated estrone fraction from urine and of the pure estrone-3-methyl-ether, using the specific Kober reagent, make it highly probable that the substance isolated in this fraction is estrone.

The increase observed in the estriol fraction during pregnancy (table 1) can not alone be taken as evidence that estriol is excreted in the urine of the pig. The color intensity developed with the Kober reagent is considerably lower for estriol than for estrone and estradiol. Accordingly, only weak color intensities correspond to the figures given for estriol. In addition, the "background absorption" for the estriol fraction is considerably greater than for the other two fractions when pigs' urine is analyzed. More work is necessary to reach a final conclusion regarding this fraction.

In the estradiol fraction, no significant increase is observed during pregnancy.


Methylated estrone fraction from pregnant sow's urine.
25 μg. pure estrone-3-methyl-ether added to non-pregnant sow's urine.
40 μg. pure estrone-3-methyl-ether added to non-pregnant sow's urine.
Corrected Optical Density.
0,700
0,600
0,500
0,400
0,300
0,200
0,100
0,000
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Fraction numbers

Graph 1—Chromatography on alumina of methylated estrone fraction from pregnant sows' urine and of methylated estrone fractions from nonpregnant sows' urine to which pure estrone-3-methyl-ether had been added before chromatography. Solvents used were 12 ml. of 25 per cent benzene in n-hexane (discarded), and 40 per cent benzene in n-hexane. Effluent was collected in fractions; each fraction represents 1.15 ml.



Graph 2—Absorption spectra of methylated estrone fraction from pregnant sows' urine and of pure estrone-3-methyl-ether in Kober reagent as modified by Bauld.

However, studies on the optimum conditions for hydrolysis of pregnant sows' urine seem to indicate that more estradiol is liberated when longer boiling periods are used. Investigations on this point are being continued.

The results indicate that, in the sow, estrone is the main estrogenic hormone excreted in late pregnancy, and that this hormone is excreted almost exclusively in conjugated form.

Much work needs to be done to obtain information on the quantitative aspects of estrogen excretion in the nonpregnant and pregnant sow. However, the new methods developed for chemical determination of human urinary estrogens[1,2] also may be successfully applied for determination of urinary estrogens of pigs, and may prove valuable in the study of normal and pathological conditions.

SUMMARY

Using the chemical method of Brown,[2] investigations on the nature of estrogenic hormones in the urine of the sow in late pregnancy strongly indicate that estrone is the main estrogenic hormone excreted by this species. The hormone is excreted almost exclusively in conjugated form.

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The Comparative Histopathology of Rickets and an Osteodystrophy in Immature Iowa Swine

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DURING ROUTINE necropsy examination of pigs in the Iowa Veterinary Medical Diagnostic Laboratory, it was noticed that many specimens showed an osteopathy. These lesions were evident as an increased pliability of the rib shafts and, in many instances, an enlargement of the distal epiphyses was also present. Based on clinical descriptions, these lesions would be referred to as "rickets." Tissue sections from these ribs failed to show typical histological changes of rickets, as described by various authors.[2, 14, 16] Because of this, the ribs were examined to determine what changes were present.

Although the pigs used in this study were submitted to the laboratory for various reasons, they all showed similar rib lesions. Since the microscopic changes in the ribs were unknown, the determination of the cause was not attempted and the scope of this study was limited to the histological picture observed.

REVIEW OF LITERATURE

References on the occurrence of so-called rickets in swine are numerous. Many of these[7, 18–20] referred to osteopathic conditions in swine as "rickets"; however, in each case an adequate histological confirmation was absent.

Schoek[18] described research on the calcium: phosphorus ratio needed in the feed to prevent gross bone deformities in swine, which were called rickets. Elliot *et al.*[7] gave formulas for a number of rations that would prevent clinical signs associated with rickets. Sinclair[20] used rations that would prevent "stiffness" in pigs. He doubted whether the conditions were true rickets. Shaw[19] conducted experiments with pigs to determine whether glass-filtered or direct sunlight would best prevent rickets in pigs. The conclusions of the experiment were based on the rapidity of gain and the absence of clinical bone deformities. Rickets was diagnosed clinically.

Joest and Zumpe[9] expressed doubt that rickets occurs in domesticated animals, and Neiberle and Cohrs[12] stated that rickets does not occur in domesticated animals.

Hutyra *et al.*[8] stated in reference to rickets: "The disease usually occurs in young animals (under 1 year old), especially soon after weaning, but may occur at any stage of growth. It is most common in young pigs, puppies, lambs and kids, rarer in colts, calves and rabbits." They included a photomicrograph of the radius of a 7-month-old pig that showed lesions usually associated with rickets.

McGowan[10] reported experiments in which he produced rickets in pigs. Pigs fed a calcium-deficient ration developed osteoporosis which could not be cured by the administration of cod-liver oil, vitamin D, and sunlight. A "balancing" of the phosphorus pentoxide with the calcium carbonate failed to prevent rickets and actually aggravated the condition. He concluded that rickets was due to a relative deficiency of both calcium and phosphorus. Photographs showed histological changes that appeared to be those of rickets.

Obel[13] described rickets in 13 calves, 2 to 4 months old. She included a detailed histological description and a number of photomicrographs. A diagnosis of uncomplicated rickets was made for 8 calves, and osteoporosis was also present in 7. Although rickets was present, she attributed death to other conditions, such as intoxication from rumen "indigestion" and anemia.

Theiler,[21] in discussing rickets and osteomalacia in domestic animals, stated that there is no difference, pathologically, between the two diseases, and that a superabundance of osteoid tissue is found in both. He further states: "In veterinary medicine the two terms have also been used to describe a pathological condition in young and in adult animals. Rickets is referred to as a disease of foals, calves, lambs, kids, young pigs and puppies, and osteomalacia as a disease of horses, cows, sheep and goats; less often of pigs and dogs." By experimentally feeding heifers on a phosphorus-deficient ration for over two years, Theiler[21] was able to produce pathological bone changes. He referred to this condition as "rickets," and described an excessive formation of osteoid tissue.

Dunne *et al.*[6] described rib changes in swine affected with hog cholera. In acute cases of hog cholera, hemorrhage was present at the epiphyseal line, which was widened and irregular. In pigs affected with chronic hog cholera, transverse solid lines of bone structures were found below the costochondral junction. Microscopically, they found an enlarged area of mature cartilage cells and irregular trabecular bone. Ribs from pigs with chronic cases showed an ossified transverse area in the spongy bone. Although they had not observed these lesions in swine affected with other

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diseases, they did not consider the changes pathognomonic for hog cholera.

MATERIALS AND METHODS

The specimens used were obtained from Iowa farm swine submitted to the Iowa Veterinary Medical Diagnostic Laboratory. Tissue sections were from pigs showing marked pliability of the rib shafts and enlargement of the distal epiphyses. These specimens represented 48 swine from 47 herds. Two of the pigs were experimentally infected with hog cholera virus. With few exceptions, the pigs weighed from 20 to 60 lb. Larger pigs were included if gross rib lesions were present.

In all cases, the specimens were cut from the fourth rib, using a jeweler's saw blade with 64 teeth per inch. The rib block was limited to a thickness of 3 mm. Although the length varied, it included the distal cartilage, the zone of primary ossification and a part of the rib shaft. In most instances, a section 2 to 3 cm. in length was adequate.

The tissue specimens were fixed in 10 per cent neutral formalin-physiological saline solution for a minimum of 72 hours. The blocks were decalcified with the procedure described by Dotti *et al.*,[6] using 30 per cent formic acid and ammonium salt

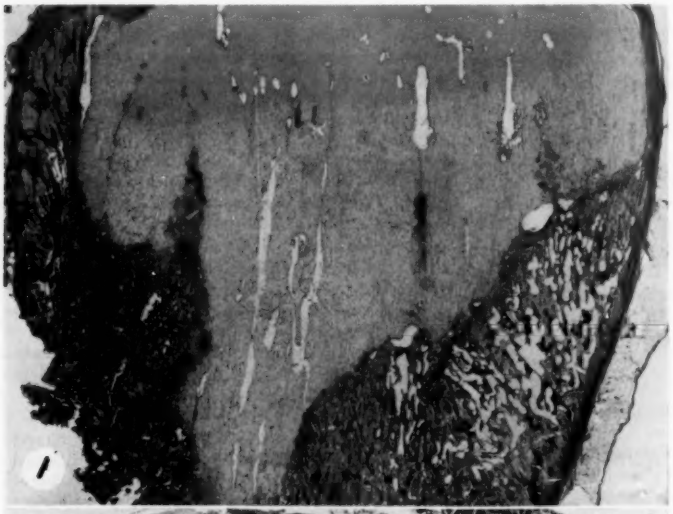

Fig. 1—Photomicrograph of the distal rib epiphysis of a pig. The zone of columns and the vesicular zone are greatly widened, and the epiphyseal line is irregular. x 5.5.

Fig. 2—Photomicrograph of the distal rib epiphysis, showing lesions similar to those in figure 1. The cartilage zones are widened and the epiphyseal line is uneven. x 4.7.

of sulfonated resin (WIN-3000*). Decalcification was complete in three to five days. This technique resulted in good preservation of cytological detail. The tissues were washed in running tap water overnight, then dehydrated with 70 per cent, 95 per cent, and absolute ethyl alcohol. They were cleared in chloroform and embedded in Altman's paraffin-stearin-beeswax mixture. The embedded tissues were sectioned at 7 μ.

Two staining techniques were used: (1) the sections were stained routinely with Mayer's hematoxylin and counterstained with ethyl eosin, except that the time in hematoxylin and tap water was increased to five minutes each. (2) A modified Hansen-Bock stain [11] also was used to distinguish between osteoid tissue and calcified bone. This stain provided better differentiation of the cellular elements than the ordinary hematoxylin and eosin method.

RESULTS

The tissue blocks were examined grossly when they were removed at necropsy. The epiphyseal line in most of the ribs was even; however, marked irregularities were observed (fig 1, 2). Some of the ribs showed hemorrhage at the distal epiphysis, which was widened in relation to the shaft.

With low magnification (x 12), a few tissue sections showed marked irregularities of the epiphyseal line. When osteoid tissue was present, it was found as a thin layer on the trabeculae. In some sections, the trabeculae of the spongiosa were thin and fully formed, indicating osteoporosis. The cartilage was not excessively vascular.

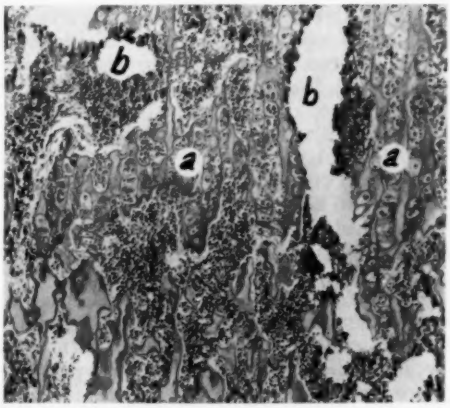

Fig. 3—Photomicrograph of an area in the primary spongiosa, of a pig, containing uncalcified and necrotic cartilage (a). The trabeculae are distorted. Hyperemia is indicated by dilated (b) sinusoids (blood was lost during tissue sectioning). Osteoblasts are almost completely absent. x 77.

Hemorrhage at the epiphyseal line was evident in some of the sections, and hyperemia of the bone marrow in the primary and secondary spongiosa was found in other sections.

With higher magnifications, the lesions observed in a majority of the sections followed a pattern.† The zone of columns and

* Manufactured by Winthrop Laboratories, 1450 Broadway, New York 18, N.Y.

† A detailed description of the tissue sections can be found in a thesis entitled, "Suppressed Endothelial Cell Differentiation in the Ribs of Immature Iowa Swine," by Aaron H. Groth, Jr., Iowa State College Library, Ames, 1957.

Fig. 4—Photomicrograph of an area showing hemorrhage along the epiphyseal line (most of the blood was lost during tissue sectioning). The diagnosis in this case was hog cholera. x 96.

the vesicular zone of the cartilage was increased in width. The epiphyseal line was irregular and the cartilage breakdown was suppressed in many of the sections. The primary spongiosa contained distorted trabeculae and islands of uncalcified and necrotic cartilage (fig. 3). Many cartilage cells stained red with eosin and their nuclei were pyknotic.

In most sections, the osteoblasts were almost completely absent or were of the inactive type. Pritchard [15] described an active osteoblast as one that had a cuboidal or columnar shape, while the inactive osteoblasts assumed the squamous form. In some of the rib sections, active osteoblasts were present, but were confined to small areas.

In the sections from 4 pigs, large amounts of blood were present at the epiphyseal line, indicating hemorrhage (fig. 4, 5). Where this was present, the cartilage breakdown did not occur. It appeared that this line of hemorrhage prevented capillary invasion of the cartilage.

There was hyperemia of the bone marrow in many sections and numerous multinucleated cells were present. These cells were not adjacent to the bone trabeculae, but were scattered throughout the bone marrow substance. They contained more nuclei than are usually found in megakaryocytes, and resembled tissue giant cells. Hypoplasia of the bone marrow was noticeable in many sections, with only the reticular network remaining. In the sections from several pigs, this area of bone marrow had been replaced by fibrocytes.

Osteoid tissue was present only as a thin layer along the trabeculae, and in many sections it was almost absent; large amounts never were found.

A secondary band of cartilage was found below the epiphyseal line in the sections from 2 pigs. In these instances, the bone formation evidently had been interrupted and then had resumed.

DISCUSSION

The major histological changes observed in the ribs may be grouped as follows: (1) a widening of both the zone of columns and the vesicular zone of the cartilage; (2) suppressed cartilage breakdown; (3) thin, distorted trabeculae with necrotic and uncalcified cartilage; and (4) aplasia and hypoplasia of the osteoblasts.

The cartilage cells of the vesicular zone appeared to be maturing, as indicated by enlargement, an increase in endoplasm, and the presence of pyknotic nuclei. Both the zone of columns and the vesicular zone were widened, the apparent result of a difference in the rate of cartilage matura-

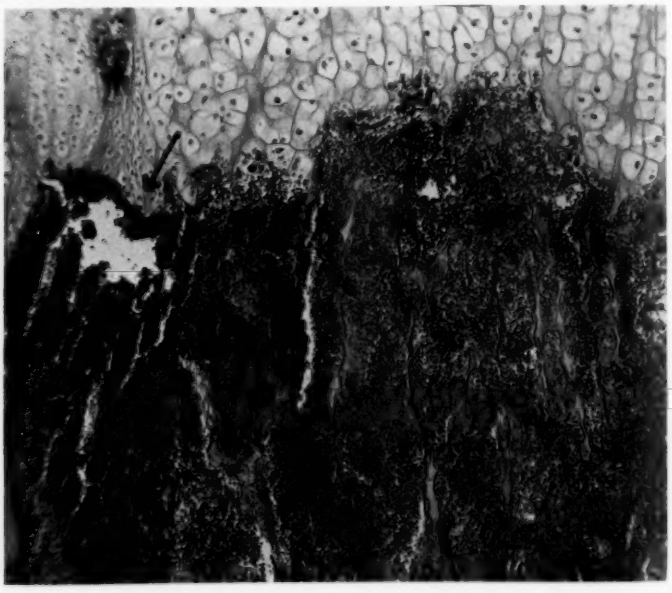

Fig. 5—An area of hemorrhage at the epiphyseal line (arrow). This pig was experimentally infected with hog cholera. x 96.

tion and breakdown. The two processes were either partially or completely independent, which would indicate the presence of a cartilage-maturing factor.

In the area of the epiphyseal line, two changes were observed. There was either hypoplasia, with little vascular endothelium, or hyperemia. In neither instance did cartilage breakdown appear to be taking place and, in some sections, a vascular channel lay perpendicular to the long axis of the bone, occupying the zone of cartilage breakdown. The cell walls of the mature cartilage were intact. Islands of necrotic and uncalcified cartilage were present in the primary spongiosa, which indicated faulty cartilage breakdown and inactivity of the osteoblasts.

In 2 pigs, the breakdown of cartilage apparently had stopped and later resumed as a new zone of cartilage invasion at the top of the widened vesicular zone. In these 2 pigs, the new zone formed around the nutrient vessels of the cartilage (fig. 6).

The trabeculae of the primary spongiosa were thin and distorted. Calcification appeared to be incomplete, especially in those sections in which osteoblasts were absent or of the squamous type. Osteoid tissue was present as a thin layer on the trabeculae. The distortion in some of the ribs was evidently the result of mechanical compression, as indicated by a bending of the trabeculae. The vesicular zone of the cartilage was also compressed. The misshapen trabeculae in other sections apparently were the result of uneven cartilage breakdown. Uncalcified and necrotic cartilage was present in these areas. The necrotic cartilage cells retained their shape, but the nuclei were either pyknotic or absent, and the cell wall stained red with the Hansen-Boek stain. The uncalcified cartilage retained a normal appearance.

Sections from 6 pigs showed marked widening of the vesicular zones. The cartilage maturation had continued; however, the cartilage breakdown evidently had ceased. In some sections, bone formation had resumed, as indicated by columnar-shaped osteoblasts. The line of cartilage breakdown was irregular and, in one section, a large triangular piece of cartilage was found in the primary spongiosa.

The osteoblasts were absent in the primary spongiosa if there was hemorrhage at the epiphyseal line. In some of the same sections, osteoblasts appeared in other areas. Other sections contained widespread osteoblastic hypoplasia. The absence of osteoblasts was most noticeable when the capillary invasion of the cartilage was inactive.

Clinically, the gross lesions observed in these pigs could be referred to as "rickets." Park [14] stated in his lecture before the Harvey Society that, " . . . the presence of osteoid in excess is a cardinal sign of rickets and is pathognomonic of the disease. It is the sign of the disease in osteomalacia and may be the sole sign in the older child." He further described a vascular invasion of the cartilage to the extent that wide "brushes" were formed.

None of the sections examined in this study showed excess osteoid tissue or vascular brushes in the cartilage. Osteoid tissue was present, but only as a thin layer on the trabeculae, and in some sections there was less than normally would be expected. It was evident from these histological findings that the pigs examined were not affected with rickets. The disease was characterized by suppressed differen-

Fig. 6—Photomicrograph of the cartilage at the distal epiphysis of a pig, showing secondary areas of cartilage breakdown (arrow). These areas are forming from the nutrient vessels of the cartilage. x 29.

tiation or hypoplasia of the osteoblasts, and an absence or suppression of cartilage breakdown.

To produce experimental rickets in laboratory animals, complete absence of sunlight is necessary, in addition to a vitamin D-deficient ration.[22] It would be difficult to assume that such conditions would occur on Iowa farms. Many pigs are raised on rations deficient in vitamin A and calcium but, even under the most unfavorable management conditions, they probably would be exposed to adequate sunlight. Although the amount of sunlight necessary to prevent rickets in pigs is unknown, Couch *et al.*[4] stated that one hour of direct sunlight per week was sufficient for satisfactory bone growth and feed utilization in chicks. Since pigs are not covered by heavy hair, sunlight can easily reach the skin.

Three of the pigs were known to have been fed a vitamin A-deficient ration. The lesions shown by these pigs resembled those attributed to a deficiency of vitamin A. The zone of cartilage breakdown was irregular, indicating that capillary invasion had occurred at an uneven rate. There was distortion of the trabeculae, which contained necrotic and uncalcified cartilage. Although osteoblasts were present and appeared active, the presence of necrotic cartilage would indicate inactivity of the osteoblasts at some time in the past.

Sandstedt *et al.*[17] described the histological changes in the hard tissues of foals which were deficient in vitamin A. The zone of cartilage columns was broadened and there was no sharp transition to the vesicular zone. If the deficiency was severe, both zones were narrowed because growth had stopped. The vesicular zone had an irregular structure and cartilage breakdown was not uniform. This resulted in large islands of cartilage being isolated in the primary spongiosa. Severe vitamin A deficiency resulted in an atrophy of the osteoblasts and the cells lay thinly scattered on the bony trabeculae. In the most severe cases, the osteoblasts disappeared entirely.

Other factors, such as hog cholera virus, could have been an etiological agent in the suppression of capillary invasion of the cartilage and differentiation of the osteoblasts. According to Runnells,[16] the hog cholera virus produces changes in the endothelium of the capillaries and other blood vessels. In acute cases those changes are degenerative.

Obel [13] stated, with regard to cartilage breakdown and bone formation:

> The exoplasm of the cartilage, for example the paler portion of the protoplasm that lies around the nucleus near the cartilage capsule increases in size and becomes vacuolated and in the nucleus there appear degenerative nuclear changes. In the cartilage exoplasm an amorphous calcification occurs at the same time. At this stage there occurs an ingrowth of so-called blastoma taps into the degenerate cartilage from vessels of the Haversian canals in the bone sleeve. The blastoma taps consist of swollen endothelial cells which reabsorb the walls of the cartilage capsule and enter into the cartilage cell. Each endothelial cell thereafter divides several times so that a syncytium of blastoma taps appears. Later these differentiate into endothelial cells and osteoblasts. In front of the blastoma tap an indentation appears that becomes filled with red blood cells; this is an extension of the capillary.

About the same process, Arey [1] stated:

> This destruction [cartilage cells] apparently is caused by the vascular primary marrow tissue which simultaneously invades the cartilage; some multinuclear giant cells, the so-called chondroclasts also are in evidence. The early marrow tissue arises from the inner, cellular layer of the perichondrium and burrows into the cartilage in bud-like masses. Such eruptive tissue gives rise both to osteoblasts and to the vascular marrow which occupies the early marrow cavities.

The endotheliotropic hog cholera virus is found in the blood streams of infected animals. When the virus reaches the proliferating endothelium of the blood vessels of the bone, it is possible that the vascular invasion of the cartilage would cease, as would the differentiation of the osteoblasts and the endothelial cells. The cartilage, a comparatively avascular substance with apparently independent growth control, would continue to proliferate and mature. The widened cartilage zones probably result from such a process. The osteoblast is assumed to have the function of building bone upon the calcified trabeculae.[15] If the hog cholera virus either prevents its differentiation or interferes with the activity of the osteoblasts, the reason for the rib lesions can be seen readily.

A diagnosis of hog cholera was made on necropsy of 9 of the 48 pigs studied. Two other pigs were experimentally infected with field strains of hog cholera virus and were killed for necropsy after the fever

began to subside. All 11 pigs had "typical" gross lesions of hog cholera. Sections taken from the ribs of these pigs showed either an aplasia or hypoplasia of the osteoblasts, hypoplasia of the bone marrow, necrotic cartilage in the primary spongiosa, and widening of both cartilage zones, and many showed the presence of hyperemia at the zone of cartilage breakdown. An increase of fibrocytes was noticed in the primary spongiosa of some sections. These lesions appeared to result from suppression of both capillary invasion and cartilage breakdown, suppressed differentiation of the osteoblasts, and increased permeability of the "vascular buds."

Dunne *et al.*[6] described bone changes in swine experimentally infected with hog cholera virus. They found a marked enlargement of the mature cartilage cells and irregular bony trabeculae in the ribs. There was also hemorrhage at the epiphyseal line if the pigs were affected with acute hog cholera and, if the pigs were affected with chronic hog cholera, transverse lines of solid bone were found 5 to 10 mm. proximal from the epiphyseal line.

Cohen[3] described costochondral lesions occurring in a 3-month-old child which had died of an influenzal meningitis. Both of the cartilage zones were widened and vascular penetration of the mature cartilage cells appeared to be diminished. His photomicrographs also showed distorted trabeculae and uncalcified cartilage. The lesions were similar to those observed in this study. The condition was described as being distinct from rickets, scurvy, and syphilis. Since swine influenza occurs in young pigs, the question arises if this virus might also contribute to the formation of these lesions.

Field cases of osteodystrophy in swine often are complicated by variable factors, such as hog cholera, hog cholera vaccination, swine influenza, deficient rations, or poor management; hence, controlled experimental investigation would be necessary to determine the cause or causes. It appeared to the author that the hog cholera virus, either as an infecting agent or as an immunizing agent, was the most probable cause. The lesions found in 2 pigs with active cases of hog cholera have been described. Experiments should be undertaken to determine if vaccination with virulent and modified hog cholera viruses would produce these lesions in pigs deficient in vitamin A, calcium, or both. In these experiments, pigs should be fed on a low level of vitamin A and calcium, since totally deficient rations are seldom encountered, particularly so during the summer months, when most rib lesions are observed. In light of Cohen's[3] description of the costochondral lesions in influenzal meningitis in man, swine influenza should be considered as a possible factor in this disease.

Summary and Conclusions

1) Tissue sections, made from the distal epiphysis of the ribs of 48 swine from 47 Iowa herds, showed an osteopathy indicated by increased pliability of the rib shaft and enlargement of the distal rib epiphysis. Rib sections from 2 pigs experimentally infected with hog cholera virus were included.

2) Examination revealed a widening of the zone of columns and the vesicular zone of the cartilage, suppressed cartilage breakdown, thin distorted trabeculae in the primary spongiosa that contained necrotic and uncalcified cartilage, and aplasia and hypoplasia of the osteoblasts. In many sections, there was hypoplasia and, in some instances, hyperemia of the bone marrow. The rib sections from the pigs infected with hog cholera showed hemorrhage at the zone of cartilage breakdown, in addition to the other lesions.

3) The histological lesions did not resemble those described as occurring in rickets. There was no excess of osteoid tissue and the cartilage lacked vascular "brushes." It is possible that these pigs were fed deficient rations; however, most of the cases were observed in the summer when there probably would be enough sunlight to prevent rickets, even under unfavorable management conditions.

4) The histological picture suggests the presence of an agent that prevented or suppressed cartilage breakdown, causing either a hypoplasia or suppressed differentiation of the osteoblasts, and an increase in the permeability of the "vascular buds."

5) Hog cholera virus or swine influenza virus are suggested as possible etiological agents. Hog cholera virus, either as an infecting agent or as an immunizing agent, could produce the bone lesions. It also is suggested that the production of the disease by hog cholera virus may be dependent upon a partial vitamin A or calcium

deficiency, or both. Similar rib lesions have been reported in human beings suffering from influenza, suggesting that swine influenza virus may be a possible etiological agent.

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Canine Mastocytoma—A Report of One Hundred Cases

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THE MAST CELL, or mastocyte, is one of the cellular components of connective tissue, together with fibrocytes, histiocytes, and plasma cells. The mast cell is found throughout the body, particularly in great numbers around small vessels in the canine skin and liver. In the loose connective tissue of the corium and subcutis, the nontumerous mast cell has a pleomorphic shape, ranging from fusiform to spherical and stelliform. The nucleus is round or ovoid and will never take on the lobulated form of the basophilic granulocyte. The mast cell is characterized by an abundance of cytoplasmic metachromatic granules which contain three biologically active substances: heparin, histamine, and hyaluronic acid. As an approach to understanding the complex biochemistry of the mast cell, much interest has been focused on the hyperplasia and neoplasia of the cell. Tumorous proliferation of mast cells frequently occurs in the skin of dogs, while it is extremely rare in other species of animals, and in man.

REVIEW OF LITERATURE

The discovery of the mast cell is attributed to Ehrlich,[25] who (in 1879) gave the first description of a connective tissue cell with characteristic metachromatic granules in its cytoplasm. He called it the "mast cell," because he had associated its presence with edematous tissue which was rich in plasma, such as in elephantiasis caused by lymphatic or venous obstruction.[25] "Mast" is the German word for feed, and the literal translation of "mast cell" is: a well-fed cell. Because of its predominantly perivascular arrangement, both the genesis and function of the mast cell have been associated with blood vessels. The origin of the mast cell is not known with certainty. However, it is a histiogenic cell,[28] and not a myeloid cell, as is the basophilic leukocyte. Perivascular mesenchymal cells were found to differentiate into mast cells when observed in studies of intact tissue spreads of rats.[59]

Biochemical Properties.—Three biologically active substances are associated with the mast cell: heparin, histamine and hyaluronic acid. Jorpes and Holmgren[30, 32] (in 1937) discovered the relationship between mast cells and an anticoagulant (a sulfuric mucopolysacharide), which they named "heparin" because of its abundance in liver tissue. Asboe-Hansen[2] (in 1953) injected radioactive sulfur (S^{35}) into mice with experimental skin tumors. Magnusson and Larsson[41] (in 1955) and Larsson[37] (in 1956) also employed S^{35} in a dog with mastocytoma. Parallel sections for autoradiography, staining with toluidine blue, and chemical heparin extractions were made. The results provided further evidence that normal and neoplastic mast cells manufacture heparin.

Riley[57] (in 1952), using the extraction method and (in 1953) the fluorescent diamidines as histamine liberators,[60] demonstrated that the mast cell contained histamine in addition to heparin. Ehrich[20] (in 1953) showed that the free-circulating basophilic granulocytes in the blood stream contained histamine, and thus resembled the tissue mast cell in function. Compound 48/80 has been used as a histamine liberator in rats by Riley and West[60] (in 1955), and in dogs with mastocytomas by Larsson[36] (in 1957), to demonstrate the presence of histamine in normal rat mast cells and in neoplastic canine mast cells. Asboe-Hansen[2] (in 1951) emphasized the preponderance of mast cells in loose fibrillar connective tissue and serous membranes. He postulated that a heparin-like substance is secreted by the mast cell and is desulfated into hyaluronic acid. Romanelli[46] (in 1953) found that neoplastic mast cells from a canine mastocytoma contained hyaluronic acid as well as heparin. Symmers and Thomson[71] found hyaluronic acid and heparin in mast cell lesions of a ferret.

Disturbance of Growth in Man.—Mast cell hyperplasia of human skin has long been recognized as a pathognomonic lesion in urticaria pigmentosa, and has also been seen in the periphery of many neoplasms. Ellis[23] (in 1949) reported an infant with urticaria pigmentosa. Mast cell hyperplasia was also present in liver, spleen, bone marrow, and mesenteric nodes. The author drew attention to the similarity of this case to mastocytoma of dogs. Hissard *et al.*[28] (in 1950) described cutaneous mastocytosis in a 47-year-old man, which began three years previously as urticaria. Several ulcerated, firm, and painless nodules were present in the abdominal and gluteal skin, and there was hepato-splenomegaly. A cutaneous nodule con-

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TABLE 1—One Hundred Canine Mastocytomas from the Collection of the Department of Veterinary Pathology at the Ohio State University

Breed	Sex	Age (yr.)	Size (cm.)	Site	Remarks
.....	....	....	Large	Groin Hip	Ulceration, immature, developed rapidly; inguinal node metastasis.
.....	....	....	.....	Hock	Closely packed, immature cells.
Chow	F	6	7 x 3	Flank	Ulceration; diffuse, loose, lymphatic invasion.
Boston Terrier	M	7	2 x 1	.....	Ulceration, duration 3 mo., immature.
German Shepherd	M	8	Large	Groin Multiple	Ulceration, loose, immature cells; recurred.
Boston Terrier	F	8	12 x 12	Lumbar area	Ulceration, recurred in 8 mo., fine granules.
Coon Hound	M	4	10 x 10	Right flank	Duration 1 mo., mature, recurred in 4 mo.
Boston Terrier	F	8	3 x 3	Shoulder	Mature, diffuse; many eosinophils.
Irish Terrier	F	11	2 x 2	Multiple Vulva & thigh	Ulceration; loose, invasive, edema.
Am. Bull Terrier	M	10	.....	Scrotum	Diffuse in corium; mature cells.
Boxer	M	2	1 x 1	Head	Diffuse infiltration, mature cells.
Terrier X	F	7	.....	Entire thigh Multiple	Diffuse infiltration, dustlike granules; recurrence.
Boston Terrier	F	5	.....	Axilla	Duration, 1 mo.; infiltration of striated muscle.
Cocker Spaniel	M	5	.....	Footpad	Loose, mature cells; recurred in 2 weeks.
Boston Terrier	F	6	Large	Foreleg	Loose, immature; previous recurrences; metastasis to spleen, liver.
Fox Terrier	F	12	5 x 3	Vulva	Diffuse, packed, immature cells.
.....	SF	5	5 x 4	Multiple Trunk	Mature cells in corium.
Spitz	M	12	6 x 4	Foreleg	Mature, between muscle fibers; eosinophils, giant cells.
Mongrel	M	7	.....	Thigh Multiple	Duration 2 mo.; immature, loose, mitoses.
Pointer	M	6	5 x 4	Submax.	Duration 3 mo.; mature, many eosinophils.
Boston Terrier	M	10	.....	Flank	Duration 6 mo.; closely packed, distinct granules seen in H and E.
Scottish Terrier	M	6	4 x 4	Scrotum	Immature, diffuse, many eosinophils.
Terrier X	F	3	4 x 3	Vulva	Ulcerated; mature, closely packed, eosinophils.
Chow	F	16	6 x 5	Thigh and shoulder Multiple	Ulcerated, rapid growth, neutrophils, recurred in 4 mo.
Scottish Terrier	F	6	3 x 2 2 x 2	Multiple Chest and front leg	Immature, metastasis to axillary node and liver; recurred in 2 mo.
Am. Bull Terrier	F	7	3 x 2	Chest	Duration 10 mo.; diffuse, loose, mature.
Boxer	M	3	.....	Prepuce	Duration 3 mo.; mature.
Boxer	SF	8	.....	.....	Duration 2 mo.; mature, edema.
Boxer	F	7	1 x 5	Multiple Hindleg	Ulceration, immature, packed giant cells.
Boston Terrier	M	8	6 x 6 3 x 4	Elbow Neck	Immature, packed, recurred in 3 mo.

TABLE 1 (Continued)—One Hundred Canine Mastocytomas from the Collection of the Department of Veterinary Pathology at the Ohio State University

Breed	Sex	Age (yr.)	Size (cm.)	Site	Remarks
Collie	M	13	6 x 4	Radius	Uniform, large, immature; numerous mitoses.
Boxer	M	3	7 x 7	Shoulder	Mature, many neutrophils.
Basset Hound	M	6	6 x 6	Scrotum	Mature, many eosinophils.
Boston Terrier	M	9	5 x 4	Scrotum	Duration 4 mo.; giant cells, many eosinophils.
Walker Hound	M	10	14 x 14	Neck	Granular, mitoses; metastasis to liver, lung, spleen.
Fox Terrier	F	10	1 x 1	Eyelid	Mature, closely packed.
Boxer	M	....	4 x 3	Scrotum	Packed, mature; recurred in 4 mo.
Boston Terrier	M	10	1 x 1	Trunk Multiple	Immature, mitoses; metastasis to sublumbar nodes, small intestine.
Mongrel	M	8	1 x 1	Thigh	Duration 6 mo., immature, metastasis to inguinal node.
Terrier X	M	1	3 x 3	Leg	Mature cells in corium and subcutis.
Pointer	M	8	2 x 1	Stifle Multiple	Mature, loose, edema.
Dachshund	F	13	1 x 1	Abdomen Multiple	Ulceration, mature; recurred in 2 mo.
English Bull	M	3	6 x 2	Groin and thigh Multiple	Ulceration, fine granules, diffuse, loose.
Boxer	M	....	4 x 4	Stifle	Mature, eosinophils.
Boston Terrier	....	13	5 x 5	Shoulder	Ulceration, immature, dustlike particles; recurred in 1 yr.
Boston Terrier	M	9	Large	Hip	Duration 6 mo.; mature.
.....	....	....	Large	Scrotum	Immature, loose, neutrophils; many mitoses.
Collie	M	5	8 x 8	Groin	Immature, recurred in 2 mo.; metastasis to inguinal and lumbar nodes.
Boston Terrier	M	....	.....	Scrotum	Duration 1 mo.; immature, packed.
Boston Terrier	F	7	4 x 2	.....	Mature, few granules; fibrosis.
Fox Terrier	....	....	.....	Hindleg	Mature, neutrophils.
Terrier X	M	....	6 x 4	Scrotum	Immature, diffuse; inguinal node metastasis.
Boxer	....	....	.....	Scrotum	Closely packed, granular; metastasis to inguinal node.
Boston Terrier	F	4	.....	Mutiple	Mature mast cells.
Terrier X	M	7	2 x 2	Hock	Duration 1 yr; mature, large granules.
Beagle	M	9	Large	Hip Multiple	Diffuse infiltration, immature; necrosis.
Springer Spaniel	F	6	3 x 3	Foreleg	Ulceration, closely packed, few granules; lymph node metastasis.
Boxer	M	7	3 x 2	Nose	Immature, packed; removed 10 mo. ago.
Boston Terrier	....	8	.....	Hindleg	Recurred in 6 mo., immature, packed.
Cocker Spaniel	F	9	7 x 4	Left thigh	Mature, diffuse between collagen fibers.
Boxer	M	5	2 x 2	Scrotum	Diffuse, infiltration, immature.
Boxer	M	4	2 x 2	Stifle	Ulcerated, growth slow for 5 mo., then rapid.

TABLE 1 (Continued)—One Hundred Canine Mastocytomas from the Collection of the Department of Veterinary Pathology at the Ohio State University

Breed	Sex	Age (yr.)	Size (cm.)	Site	Remarks
Boxer	M	5	3 x 2 2 x 2	Right elbow Rear foot	Mature cells, loose.
Terrier X	F	11	.....	Left humerus	Duration 3 mo., diffuse, infiltration; dustlike granules.
Boston Terrier	M	10	2 x 2	Left flank	Duration 2 wks., packed, immature; striated muscle invasion.
Cocker X	F	10	6 x 4	Right tibia	Mature, neutrophils, edema, loose.
Terrier X	F	6	3 x 2	Right hind-foot	Mature, granules visible in H & E.
Mongrel	....	....	2 x 2	Left tibia	Ulceration, dustlike granules, eosinophils.
Mongrel	M	9	4 x 3	Multiple	Dustlike granules; metastasis to prescapular node and spleen.
Boston Terrier	M	12	4 x 4	Scrotum	Mature, edema; inguinal node metastasis.
Cocker Spaniel	M	7	3 x 3	Upper lip	Ulcerated, duration 1 yr.; immature.
Cocker Spaniel	M	9	.....	Ventral Chest	Slow for 2 yrs., mature; granules visible in H and E.
Mongrel	M	11	1 x 1	Flank	Ulceration, mature mast cells.
Boxer	F	6	2 x 2	Metatarsus	Mature, diffuse infiltration, many neutrophils.
Mongrel	M	8	2 x 1	Front foot	Ulceration, diffuse, mature cells, few neutrophils.
Terrier X	M	15	6 x 4	Flank	Ulcerated, packed, immature cells, few mitoses.
.....	....	....	.....	Ventral chest	Ulcerated, few dustlike granules; recurred in 3 mo.
Terrier X	F	8	3 x 3	Hock	Duration 2 mo.; dustlike granules, neutrophils.
Mongrel	F	8	4 x 3	Flank	Ulcerated, removed 4 mo.; immature.
Cocker Spaniel	F	7	2 x 2	Hip	Duration 1 mo.; mature cells.
Am. Bull Terrier	F	9	4 x 3	Flank	Ulceration, grew rapidly for 4 mo.; packed, immature.
Terrier X	....	12	2 x 2	.....	Recurred in 4 mo.; immature cells.
Pekingese	M	1	1 x 1	Chest	Ulcerated; granules seen in H & E.
Fox Terrier	F	11	6 x 6	Perineum	Duration 6 mo., ulceration, immature.
Mongrel	F	6	4 x 3	Lateral abdomen	Infiltrating striated muscle, dustlike granules, eosinophils.
Am. Bull Terrier	M	11	4 x 3	Left stifle	Ulceration, granular cells, metastasis to inguinal node and spleen.
Boxer	F	9	2 x 2	Perineum	Ulceration, duration 6 wks.; mature, eosinophils.
Fox Terrier	F	4	4 x 4	Perineum	Duration 4 mo.; immature, few mitoses.
Boxer	M	7	2 x 2	Multiple	Ulceration; many foci of immature mast cells.
Shepherd X	SF	7	2 x 2 6 x 6	Flank Groin	Began 1 yr. ago. Began 2 mo. ago; sublumbar node and liver metastases.
Terrier X	F	9	4 x 4	Hindleg	Loose, mature mast cells.
Great Dane	F	10	8 x 7 2 x 2	Left mammary region Left cheek	Edema, loose, many neutrophils.

TABLE 1 (Concluded)—One Hundred Canine Mastocytomas from the Collection of the Department of Veterinary Pathology at the Ohio State University

Breed	Sex	Age (yr.)	Size (cm.)	Site	Remarks
Boston Terrier	F	14	3 x 2	Thigh	Foci of mature mast cells.
Boxer	M	9	4 x 2	Ear	Diffuse proliferation of mature granular cells.
Boston Terrier	F	12	4 x 4	Thigh	Ulceration, diffuse, immature.
Terrier X	F	4	4 x 3	Elbow	Diffuse proliferation, mature.
Scottish Terrier	M	13	5 x 4	Flank	Duration 1 mo., ulceration, recurred in 2 mo., metastasis to inguinal node.
Doberman	M	6	8 x 4	Shoulder	Duration 1 yr.; diffuse proliferation, mature.
Boxer	F	6	3 x 5	Groin Diffuse	Inguinal node metastasis, few granules.
Great Dane	F	11	6 x 6	Mammary region	Duration 7 mo.; few dustlike granules, occasional vacuoles.

tained 99 per cent mast cells, a bone marrow aspiration 55 per cent mast cells, and a splenic biopsy performed three months later revealed 53 per cent mast cells.

Mast Cell Tumors in Animals.—The reports of mast cell tumors are frequent in dogs and extremely rare in other animals. F. Bloom [8] cites a report of a mast cell granuloma on the lip of a horse and two other reports describing mast cell hyperplasia in the skin of mice.

Twort and Twort [72] found aggregations of mast cells in the skin of mice after tar paintings. They appeared as flat warts and were benign except in one case, in which death occurred from diffuse infiltrations in the liver and spleen.

Symmers and Thomson [71] described three spontaneous mast cell lesions in the skin of a ferret which was dying of distemper. They were considered non-neoplastic and the internal organs contained no evidence of metastasis.

Monlux [46] described an ulcerated mastocytoma of the upper lip of a cat. The tumor consisted of a dense mass of granular cells with scattered neutrophils, eosinophils, and lymphocytes.

Canine Mastocytoma.—Tumors of mast cell ori-

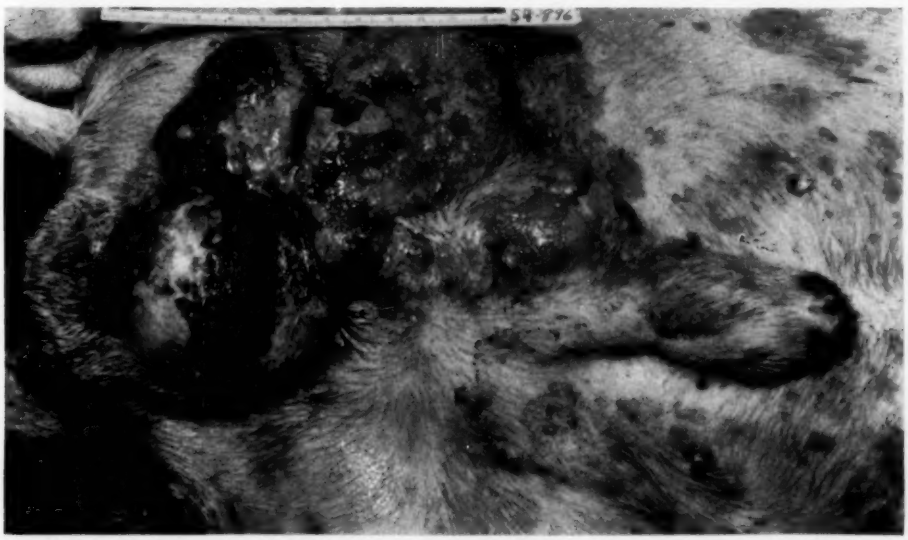

Fig. 1—Inguinal region of a dog with an ulcerating, nodular mastocytoma present in the skin of scrotum and prepuce.

gin in the skin of dogs were recognized for the first time by Murray [50] (in 1908), who diagnosed 2 cases of mast cell sarcomas (both on the hindleg) among 21 cutaneous canine tumors.

In 1931, Chambers [14] reported three male terriers with mastocytomas; in 2 dogs the hindleg was affected. He pointed out the tendency of mastocytomas to recur.

McClelland [43] (in 1940) classified one third of all cutaneous tumors of dogs as round cell sarcomas, and some of these were described as myeloid in type. Four of them recurred and two metastasized.

F. Bloom [9] (in 1942) presented the first detailed pathological description of this tumor and suggested the term mastocytoma. Among 49 primary cutaneous tumors, five mastocytomas were found, two of which were of multiple occurrence. One dog had foci of tumorous mast cells present in liver, spleen, bone marrow, and lung. Rod-shaped crystalloids and spherical bodies were present in many neoplastic mast cells.

In 1944, Jackson [31] stated that mast cell tumors in the skin of dogs occur more frequently than is suspected, and when fixation is poor they are difficult to distinguish from the contagious venereal tumor.

Oliver *et al.*[52] (in 1947) reported 15 dogs with mastocytomas, and studied in particular the extraction of heparin. The tumors, which had mature and heavily granular mast cells, were found to have a heparin content 50 times higher than that of normal dog liver. In a less mature mastocytoma, with few and dustlike granules, the heparin content was 1.7 times higher than that of liver. Repeated attempts to transmit the tumor to other dogs were unsuccessful.

Paff *et al.*[54] (in 1947), in a report on tissue cultures of neoplastic mast cells, described the malignant features of a mastocytoma in a dog. After removal of a solitary mastocytoma on the hip of a 9-year-old male Boston Terrier, multiple cutaneous nodules developed with metastasis to lung, liver, and spleen.

In 1948, Willis and Rudduck [73] described a multiple basophil-cell tumor in a 14-year-old male Pug, and a solitary basophil tumor of the scrotum of a 12-year-old English Setter. The histological description and illustrations are compatible with a diagnosis of mastocytoma.

Mulligan [46] (in 1948) reported 47 cases, 4 of which were of multiple occurrence. Seven of 10 dogs with mastocytomas, studied at necropsy,

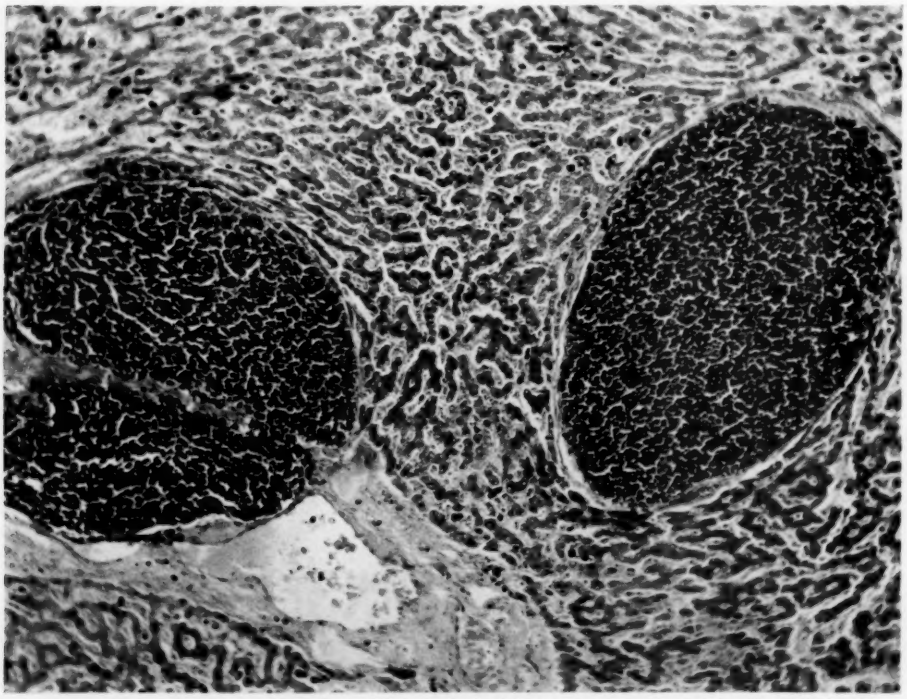

Fig. 2—Liver of a dog with two large discrete foci of tumorous, granular mast cells. Individual normal mast cells are scattered between the hepatic cords. Giemsa's stain. x 125.

TABLE 2—Age Distribution of 500 Consecutive Canine Cases Admitted to the Clinic, in July, 1956, and in January, 1957, and of 89 of the 100 Dogs with Mastocytomas (Age of 11 Unknown)

Age in years	All clinical cases		Mastocytoma cases	
	Number	Percentage	Number	Percentage
1 or less	198	39.6	2	2.2
2	57	11.4	1	1.1
3	50	10.0	4	4.5
4	50	10.0	5	5.6
5	37	7.4	6	6.8
6	27	5.4	12	13.5
7	13	2.6	12	13.5
8	24	4.8	11	12.4
9	8	1.6	10	11.3
10	10	2.0	8	9.0
11	9	1.8	6	6.7
12	6	1.2	5	5.6
13	4	0.8	4	4.5
14	3	0.6	1	1.1
15	1	0.2	1	1.1
16	3	0.6	1	1.1

showed lymph node metastasis; 19 cases were found in Boston Terriers.

Hauser [39] (in 1948) found 12 mast cell tumors during a five-year period, two of which recurred, while one metastasized to the regional lymph nodes. The author was uncertain about whether the mast cell tumor is an inflammatory or neoplastic process.

In 1949, Rubarth [67] described the histopathology of mastocytomas in 4 dogs in Sweden.

Mulligan [49] (in 1949), in his book on canine neoplasms, added 31 mast cell tumors to his previous publication of 47 tumors. Of these, 30 per cent were located on the hindlegs and 6 per cent of the dogs had multiple mast cell tumors.

Hangartner [25] (in 1949) described 11 dogs with mastocytomas under the diagnosis of basophilic granulomas. He stated that the tumors were never malignant, and that both the cutaneous mastocytoma and its metastases were to be considered as infectious granulomas.

In 1949, Ottosen [58] studied 201 cutaneous canine tumors and found 101 sarcomas. On the basis of the clinical and pathological descriptions, it appears that, in two categories, the majority of tumors were actually mast cell tumors. These two categories were named "reticulosarcomas," of which 67 cases were found, and "sarcomalike R.E.S. proliferations," of which 8 cases were encountered.

F. Bloom [7] (in 1952) treated a dog with multiple mastocytomas with cortisone at the rate of 100 mg. daily for nine days. Repeated biopsies of the nodules revealed vacuolization of the cytoplasm. After nine days, the tumors had disappeared macroscopically.

Nielsen [51] (in 1952) described the clinical behavior of 50 mastocytomas, 31 of which occurred on the thigh and external genitalia. The Boston Terrier and the Boxer showed a definite breed susceptibility. Sex was of no significance. Half of the dogs were between the ages of 5 and 9 years, and 13 dogs had multiple mastocytomas. Lymph node metastases were present in 13, and 3 of 5 dogs necropsied showed metastases in internal organs. After surgical excision, 17 cases recurred. Treatment with cortisone, following the outline and dosage recommended by F. Bloom,[7] did not result in the recovery of 1 dog with multiple mastocytoma, or another with a recurrent solitary mastocytoma.

In 1953, Riley and West [58] made histamine assays of two canine mast cell tumors. A 5-year-old female Boxer showed 295 μg. per gram, and a 9-year-old male Irish Setter had 103 μg. of histamine per gram of tumor tissue.

Brodey *et al.*[10] (in 1953) treated 3 dogs unsuccessfully with cortisone. In a 5-year-old male Pointer with multiple tumors and axillary node metastasis, after an initial reduction in size, a decrease in dosage was followed by marked increase in the size of the tumors.

Romanelli and Dozza [64] (in 1953) gave a detailed morphological and histochemical description of a mastocytoma in the popliteal region of a 4-year-old male Pointer.

In 1953, Head [27] found 29 mast cell sarcomas among 709 skin tumors encountered in a 12-year period. After surgical removal, three recurred, two metastasized to the regional lymph nodes, and two were of multiple occurrence.

Cass *et al.*[13] (in 1953) extracted heparin and histamine from six canine mastocytomas (3 of which had recurred), and compared it with the

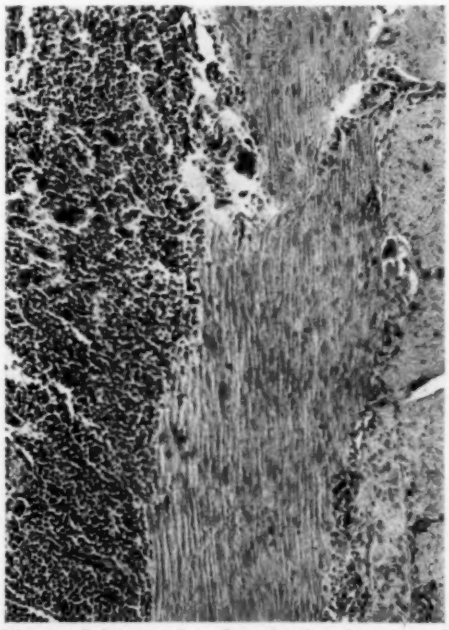

Fig. 3—Jejenum of a dog showing invasion of the longitudinal layer of the muscularis with neoplastic mast cells. H & E stain; x 100.

TABLE 3—Breed Distribution of 82 Dogs with Mastocytomas (Breed Unknown on Remaining 18)

19	Boston Terriers	4	Am. Bull Terriers
17	Boxers	3	Scottish Terriers
12	Terrier mongrels	3	Hounds
8	Mongrels	2	Chows
7	Cocker Spaniels	2	Collies
5	Fox Terriers		

heparin and histamine content of normal canine viscera. Much higher values per gram of tissue were present in the mastocytomas, particularly of histamine. It was concluded that metastasis of a mastocytoma can be anticipated by the presence of raised histamine and heparin levels of the organs.

Cotchin [16, 17] (in 1954) reported 23 mast cell lesions among 932 cutaneous tumors found in a 14-year period. After surgical removal four recurred, and 1 dog showed metastases to the iliac and inguinal lymph nodes at necropsy. It was mentioned that the neoplastic nature of mast cells is still doubtful.

In 1954, Köhler [24] reported 5 cases, and presented reasons for mastocytomas being benign granulomas. Hyalinized collagen was found in the walls of small arteries in the tumor tissue.

Runnells [29] (in 1954) presented a brief description of the salient features of canine mastocytomas and mentioned their occasional occurrence in cats.

G. Bloom *et al.*[8] (in 1955) found that mastocytomas were relatively rare in dogs in Sweden. These tumors seemed to be benign and seldom gave rise to metastases. During 1952, 22 dogs were surgically treated for mastocytomas; 17 were Boxers. No lengthening of the coagulation time in the peripheral blood was noticed. Blood taken from the tumors after removal did not coagulate *in vitro* for 60 minutes. If capillary blood was used, these results may require further evaluation, since capillary blood ordinarily does not coagulate. Blood from the cephalic veins of the same animals clotted in three to five minutes. The coagulation time was eight to 14 minutes at 24 hours after tumor removal, and profuse bleeding from the surgical wound often occurred. This could be stopped by intramuscular injection of antihistamine (2 mg./kg. body weight).

In 1955, Stünzi [30] reported 13 canine mastocytomas seen in a three-year period. They all developed in three to eight weeks, and three recurred after excision.

G. Bloom *et al.*[9] (in 1956), in an electron-microscope study of neoplastic mast cells from canine mastocytomas, found that mast cell granules and mitochondria are separate and different structures, thus refuting the theory that the granules were specialized forms of mitochondria.

Köhler [25] (in 1956), reporting 21 mastocytomas and 9 cases of mast cell hyperplasia, stated that a distinction between them is arbitrary. The age, breed, and site incidence of the two conditions was similar.

In 1956, Larsson [26] found 35 mastocytomas among 266 cutaneous canine tumors presented in a two-year period. A statistical evaluation of this material on the basis of the normal canine population showed that the average age of the mastocytoma group was 7 years, that the sexes were equally represented, and that Boxers predominated.

Brodey [11] (in 1956) described an ulcerated scrotal mast cell sarcoma in a 10-year-old Boston Terrier. The tumor was present for six months and had grown rapidly for the last five weeks.

MATERIALS AND METHODS

The material on which this study was based consisted of 92 surgical biopsy specimens and 8 necropsy cases, which were submitted to the Department of Veterinary Pathology for diagnosis during the eight-year period from 1949 to 1957. These 100 mastocytomas constituted 20 per cent of the total number of canine cutaneous neoplasms examined during this period, excluding neoplasms of the mammary gland. The majority of cases had originated in Ohio.

To evaluate the age, sex, and breed incidence in the mastocytoma material, an attempt was made to establish the normal mother population for this group. Knowing the definite shortcomings of such an undertaking, it was decided to tabulate consecutive admissions of dogs to the Ohio State University Veterinary Clinic. It was found that 40 per cent of the dogs were admitted for reparative surgery, vaccinations, and other nonmedical and non-neoplastic diseases. As referred cases of teaching value are accepted from a wide area, the population of canine admissions approximates the population from which the tumor material was derived. To avoid the influence of a seasonal overrepresentation, the months of January and July were tabulated. In a total of 500 canine admissions, the age, sex, and breed were recorded.

The surgical biopsy specimens were received in either fresh or fixed states. The specimens were described as to location, size, color, consistency, and relation to epidermis. Impression smears were prepared from the cut surfaces of all unfixed tumors and stained with Wright's stain. All tissues submitted were fixed in 10 per cent neutral formol-saline solution and Zenker's fluid. The

TABLE 4—Breed Distribution of 500 Consecutive Canine Admissions to the Ohio State University Veterinary Clinic in July, 1956, and January, 1957

Breed	No. of dogs	Percentage
Cocker Spaniel	93	18.6
Collie	56	18.6
Terrier mongrel	53	10.6
Beagle	36	7.2
Boxer	34	6.8
Dachshund	29	5.8
Mongrel	23	4.6
German Shepherd	19	3.8
Hound	16	3.2
Boston Terrier	10	2.0
English Setter	9	1.8
Irish Setter	9	1.8
Pekingese	9	1.8
Manchester Terrier	8	1.6

Other breeds were represented by 7 or fewer dogs.

routine stain was hematoxylin-eosin. To visualize the mast cell granules and other structures the following special stains were used: Giemsa's,[51] Wright's,[51] methylene blue,[43] Unna-Pappenheimer's,[43] toluidine blue,[42] periodic acid Schiff,[44] Wilder's reticulum,[42] and Masson's and Schorr's trichrome.[24] During the course of this study, a large number of sections from old cases have been recut from the paraffin blocks and wet tissues in the department's tissue storage files.

RESULTS

Gross Description.—The cutaneous mast cell tumors were found to be poorly demarcated neoplasms originating in the dermis. The cut surface was characteristically light orange in color, and usually moist with edema fluid. The overlying epidermis was ulcerated in 30 mastocytomas, either mechanically, from licking or scratching, or from ischemia due to mast cell invasion (fig. 1). In this material, all stages of mast cell lesions were observed, from a small intradermal proliferation measuring 1 by 1 cm., to a large ulcerating, diffuse mass measuring 12 by 12 cm.

Fifteen per cent of the mastocytomas were located on the external genitalia, indicating a preference for this site. Tumors on the hindquarters accounted for approximately 50 per cent of all mastocytomas on which information as to location was available (table 1).

Evidence of malignancy in 28 dogs was indicated by metastasis in 16 and recurrence in the scar after excision in 17; both occurred in 5 animals. It is also noteworthy that 9 of the 28 dogs had multiple cutaneous mastocytomas.

The recurrences were first observed two weeks to one year after excision, as follows: two weeks—1; two months—4; three months—2; four months—4; six, eight, ten, and 12 months—1 each. The interval was uncertain in 2 cases.

Eight dogs were necropsied and all were found to have metastases in one or more of the following organs: lymph nodes, liver, spleen, jejunum, and lungs. In the remaining 8 dogs, no necropsy was performed but metastasis was present in the regional lymph nodes.

Of the 16 cases with metastases, lymph nodes were involved in 14, the spleen in 4, the liver in 4, the jejunum in 1, and the lungs in 1. They were distributed as follows: inguinal nodes only—7; inguinal nodes and spleen or liver or sublumbar nodes—1 each; and 1 each in the axillary

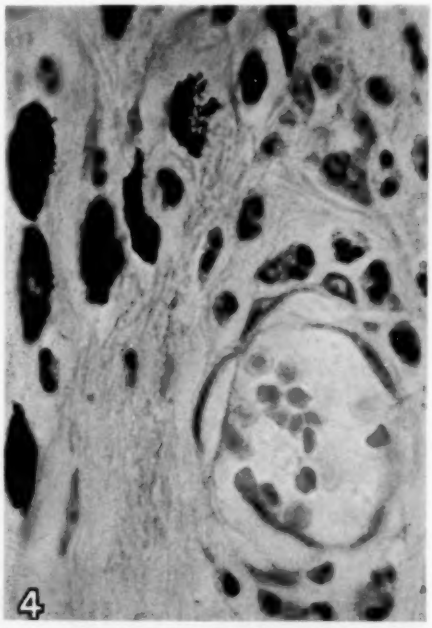

Fig. 4—Skin of a dog with dermatitis, depicting perivascular proliferation of mature mast cells and eosinophils. Giemsa's stain; x 970.

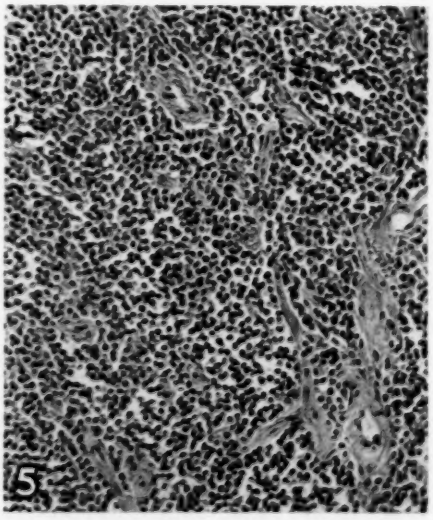

Fig. 5—Skin of dog showing a wide separation of dermal collagenous fibers by closely packed neoplastic mast cells. H & E stain; x 200.

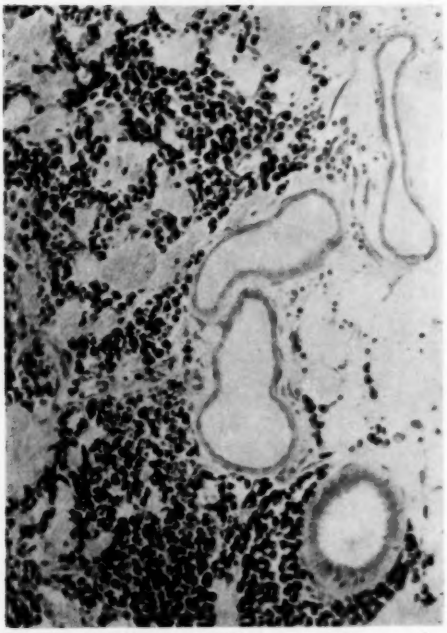

Fig. 6—Cutaneous mastocytoma with granular neoplastic mast cells surrounding apocrine sweat gland tubules. Giemsa's stain; x 200.

node alone, axillary node and liver, prescapular node and spleen, sublumbar nodes and jejunum, spleen, and liver, and in the spleen and liver, and lung.

The metastases ranged from discrete foci (fig. 2) to a diffuse infiltration of mast cells (fig. 3).

Microscopic Description.—The normal mast cells of the dermis, and those which proliferate in areas of dermatitis, were consistently pleomorphic and angular cells with abundant coarse granules, denoting maturity (fig. 4). In contrast, the tumorous mast cells were larger, round, or blunt, rectangular in shape, and their granules varied greatly in number and size, suggesting immaturity. References to mature and immature cells in this study refer to the above morphological appearance and does not necessarily reflect a gradation of anaplasia. A mastocytoma characteristically originated in the upper part of the dermis, with the proliferating cells filling out all spaces between the collagenous fibers (fig. 5). Eventually, the fibers were pushed apart, and the cutaneous glands and hair follicles were completely imbedded in mast cells (fig. 6). Tumorous mastocytes measured from 10 to 12 μ in diameter and had well outlined cytoplasmic borders. The nucleus was centrally placed, round, or ovoid, measuring from 5 to 8 μ in diameter (fig. 7). The cytoplasm, which formed a broad rim around the nucleus, was eosinophilic and occasionally showed uniform dustlike granules in hematoxylin-eosin preparations. In this study, mitotic figures were extremely rare, even in very cellular and rapidly growing tumors. Only 8 dogs showed well-formed diaster stages of mitosis (fig. 8).

Giant cells and multinucleated mast cells were also extremely rare, with only 3 tumors having this feature (fig. 9). When present, the binucleated and trinucleated giant cells were somewhat similar to the Reed-Sternberg cells of human Hodgkin's disease, although no distinct mirror image nuclei were seen. Such a mastocytoma, with immature mast cells, many eosinophils, occasional giant cells, necrosis, and fibrosis, had a morphological similarity to Hodgkin's disease.

A characteristic component of a mastocytoma was the eosinophil, which was found scattered among the mast cells, and which were occasionally present in small perivascular groups. In the mastocytomas reported here, the number of eosinophils varied greatly, and it was not possible to relate their presence to traumatization of the tumor or to the absence of mast cell granules. However, future studies may reveal a relationship between the disintegration of mast cell granules and the appearance of eosinophils.

Neutrophils and lymphocytes were occasionally present, depending on the degree of secondary inflammation. Some mastocytomas exhibited marked edema, with the mast cells spread far apart. A peculiar formation of a light eosinophilic and lacelike fibrinoid substance was observed in the wall of arterioles of a few tumors. The arteriolar wall appeared greatly thickened, with a separation of endothelium from adventitia by fibrinoid strands (fig. 10). Vacuolation of the mast cell cytoplasm was encountered in only 2 dogs. The vacuoles were circular or semicircular, forming sharply demarcated, clear areas often adjacent to the nucleus (fig. 7 and 10). Their presence could not be related to any other significant microscopic finding.

Tinctorial Properties.—In hematoxylin-eosin preparations, only a few mastocy-

tomas contained cells with visible granules. They appeared as dustlike basophilic particles distributed evenly in the cytoplasm. The metachromasia, a unique biochemical reaction of the mast cell granules, was readily demonstrated by means of toluidine blue. The intensity of the metachromasia depends on the pH of the tissue to be stained. Maximum metachromasia was seen at pH 5 and above, when all granules stained a red-purplish color.

Giemsa's stain, applied to tissue sections, rendered the granules an intense blue (fig. 2, 4, and 6), which greatly facilitated the histological recognition of immature mast cells in fast-growing mastocytomas. Certain areas of a mastocytoma often did not contain stainable granules. Modification of the technique, with a change of pH, or a switch to another dye, have not improved the staining of those areas. Wright's stain was particularly helpful when applied to impression smears of the cut surface of a tumor. This dye gives the granules a metachromatic, reddish tinge (fig. 11 and 12).

Generally speaking, mast cell hyperplasia and slowly growing mastocytomas have an abundance of coarse, deeply stained cytoplasmic granules, indicating mature cells. Mastocytomas with a history of rapid growth often have either numerous, tiny, dustlike granules, or only a few larger granules. Exceptions to this were encountered, and metastases often showed uniformly large mast cells with their cytoplasm filled with granules (fig. 2).

With Unna-Pappenheimer's methyl-green stain, the granules were a bright red which stood out on a background of a light, greenish pink cytoplasm. The granules, in a few cases of mast cell hyperplasia, gave a positive periodic acid-Schiff reaction, while granules of mastocytomas subjected to the same technique revealed only rarely a slight positive reaction. According to Drennan,[19] only certain forms of heparin (the heparin-monosulfates) will react and stain red. The highly esterified forms of heparin fail to react because their hydroxyl groups are occupied by sulfuric acid radicals.

Collagen and reticulum fibers were found not to be products of the mast cell tumor. Fibers present in a section were

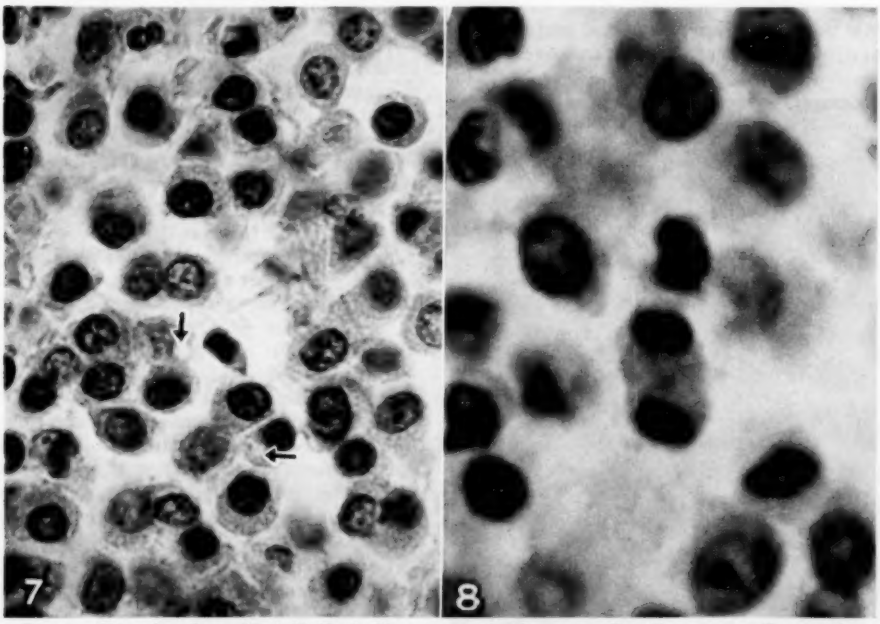

Fig. 7—Skin of a dog with individual, discrete neoplastic mast cells. Two mast cells contain cytoplasmic vacuoles. H & E stain; x 1,200.

Fig. 8—Canine dermis with freely situated, neoplastic mast cells. One diaster stage of mitosis is seen. H & E stain; x 2,200.

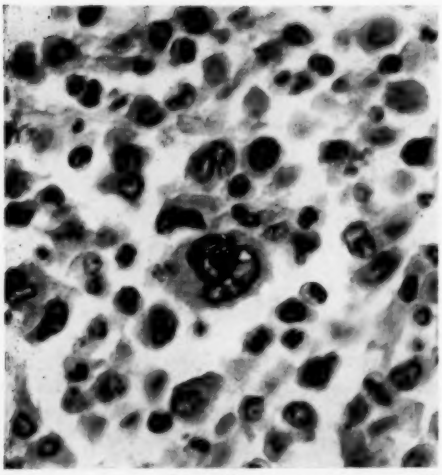

Fig. 9—Skin of a dog with pleomorphic neoplastic mast cells and a few eosinophils. One binucleated and one trinucleated giant cell can be seen. H & E stain; x 760.

attributed to the host tissue invaded by the mast cells.

Age Incidence.—The number of dogs having mastocytomas increased uniformly with increasing age, with the majority occurring at six years of age and over. This contrasted with the hospital admissions, in which the number of dogs decreased with increasing age (table 2).

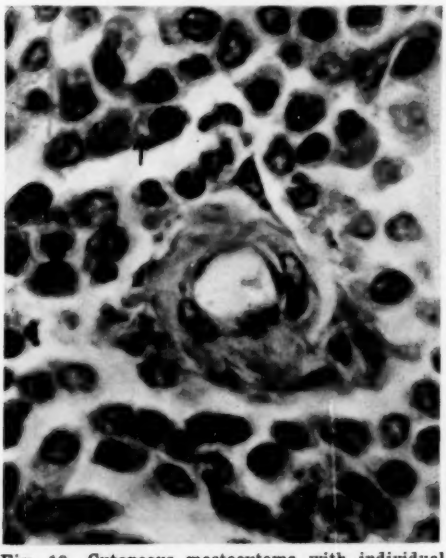

Fig. 10—Cutaneous mastocytoma with individual neoplastic mast cells surrounding a small arteriole with fibrinoid change. Note vacuolated cell. H & E stain; x 710.

Breed Incidence.—A breed predisposition was observed; 19 per cent of the dogs with mastocytomas were Boston Terriers (table 3), in contrast to only 2 per cent of the hospital admissions being Boston Terriers (table 4). The Boxer also showed a breed disposition, although not as marked as the Boston Terrier.

Sex Incidence.—This study revealed a ratio of 49 males to 41 females in the mastocytoma group, and a ratio of 230 males to 270 females in the hospital admissions. Neither group varied significantly from the biological ratio of 1 to 1. The *chi*-square for the mastocytoma group was 0.7, and for the admissions 3.2. As both values for *chi*-square were below 3.841, the variations were attributed to the methods of sampling.

DISCUSSION

A few of the lesions of canine dermatitis are characterized by edema, eosinophils, and mast cell hyperplasia. These are often interpreted as allergic reactions. Other cutaneous lesions contain an almost pure population of hyperplastic mast cells, closely resembling human urticaria pigmentosa, which is a self-limiting disease of unknown cause. In the dog, mast cell hyperplasia often results in neoplasia, while in man this is extremely rare. Only two reports [23,28] were found in which human urticaria pigmentosa gave rise to tumorous proliferation of mast cells, appearing in lymph nodes, liver, and spleen. In mice,[5,6,72] and in a ferret,[71] similar findings have been reported. In no species other than the dog has the mast cell consistently given rise to malignancy.

In this study, the term "mastocytoma" has been used exclusively. Brodey [12] preferred the classifications of "mastocytoma" and "mast cell sarcoma," while G. Larsson [39] used four different subdivisions based on morphology, and a fifth type which he reserved for recurrent mastocytomas.

Several authors [16,25,26,34] have doubted the true neoplastic nature of canine mastocytomas, and some [25,26] have considered them to be granulomas. It is often difficult to critically differentiate between hyperplasia and neoplasia since transitional forms between them are well known. The

cardinal criterion for malignancy is a growth that eventually will kill the host by invasion and metastasis. None of the dogs in this study lived long enough to succumb from the neoplasm. Many canine mastocytomas described in the literature possessed the features of a sarcoma: (1) rapid infiltrative growth of immature cells, (2) recurrence after surgical removal (often associated with rapid invasion of surrounding tissues), and (3) metastasis to the regional lymph nodes and internal organs. One or more of these criteria were present in 28 of the dogs presented in this study (table 2), thus identifying the mastocytoma with true neoplasms.

The variability of staining of the mast cell granules in mastocytomas is undoubtedly related to the chemical composition of the granules, which is believed to change with cell maturation. Drennan [19] showed that, in human urticaria pigmentosa, all of the granules contained heparin-monosulfate, which stains metachromatically with toluidine blue. After scratching, an edematous swelling develops and the mast cell granules lose their metachromasia.

Eosinophils were demonstrated by F. Bloom,[6] Hangartner,[25] Nielsen,[51], Köhler,[34] and Stünzi,[69] and were frequently found in this study. A possible explanation for the presence of eosinophils in certain areas of a mastocytoma is provided by Jadassohn.[32] He investigated human urticaria pigmentosa by taking biopsies from nonirritated lesions and from lesions that had been rubbed. The latter showed mast cells intermixed with large numbers of eosinophils, while in the former, eosinophils were absent.

The above observations by Drennan [19] and Jadassohn [32] suggest a possible relationship between trauma, the disappearance of stainable granules, and the appearance of eosinophils.

Cytoplasmic vacuoles in normal cells [15, 18] and in tumorous cells [6] have been reported, and were also seen in this study. Their significance is not known, but it has been suggested by Paff and Bloom [55] that the vacuolation is an aging phenomenon, based on their observations of vacuoles in older tissue cultures of canine tumor mast cells. Later, Paff and Mergenthaler [56] were able, experimentally, to produce cytoplasmic vacuoles in mesenteric mast cells of rats by protamine sulfate injections. This substance inactivates heparin by neutralizing its negative charges, resulting in a compensatory increased heparin production by the mast cells, and the appearance of vacuoles in their cytoplasm.

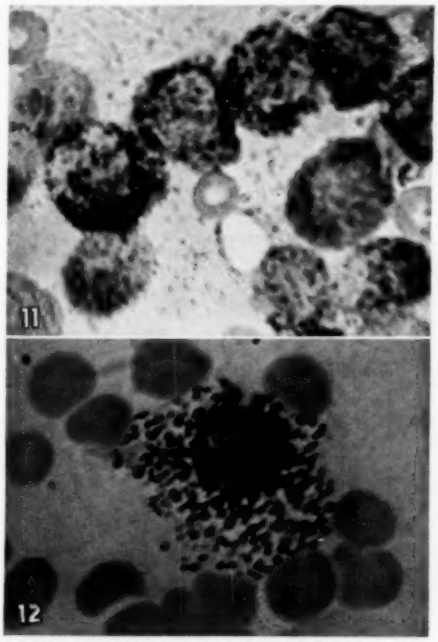

Fig. 11—Impression smear of canine mastocytoma showing erythrocytes and neoplastic mast cells with coarse cytoplasmic granules. Wright's stain; x 1,000.

Fig. 12—Impression smear showing the nucleus and granules of a neoplastic mast cell surrounded by erythrocytes. Wright's stain; x 1,440.

In this material, occasional mitotic figures were described in only 8 cases, which is in agreement with the observations of F. Bloom [6] and Köhler,[34] but contrasts with the results of Oliver *et al.*,[52] Mulligan,[48] and Stünzi,[69] who described mitoses as being fairly frequent. G. Bloom *et al.* [9] have never seen mitoses in canine mastocytomas.

A hyalinization of periarteriolar collagen in the mast cell tumor has been described by Köhler [35] and Stünzi.[69] A similar change was present in 3 per cent of the tumors in this study, and has also been seen in human urticaria pigmentosa. The walls of such vessels are thickened by loose, porous-appearing collagen. Allen [1] postulated that this reaction is caused by the

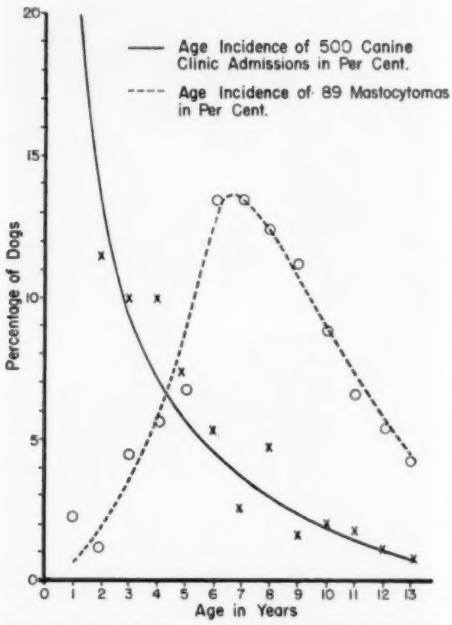
Age Incidence of 500 Canine Clinic Admissions in Per Cent.
Age Incidence of 89 Mastocytomas in Per Cent.
Percentage of Dogs
Age in Years
0
5
10
15
20
0 1 2 3 4 5 6 7 8 9 10 11 12 13

Fig. 13—Age incidence of 89 dogs with mastocytomas and 500 dogs consecutively admitted to clinic.

urticarial effect of the heparin released from mast cells by local mechanical action.

In spite of the fact that large amounts of heparin and histamine can be extracted from canine mastocytomas, an increase in coagulation time was not observed clinically by F. Bloom,[6] Oliver *et al.*,[52] and Nielsen.[51] Marked postoperative hemorrhage was found by Larsson [39] in 14 of 58 surgically treated cases of mastocytoma. This could be arrested by injection of antihistamine. He believed that the mast cells form a heparin-histamine complex, in which the heparin is rendered inactive by the histamine. It was demonstrated that the clotting time of canine blood increased with the addition of heparin, but if histamine is subsequently added, the clotting time returns to normal.

The breed incidence found in this analysis is paralleled by Mulligan's [48] findings, in the Denver area, of 19 Boston Terriers among 47 dogs with mastocytomas, and by Nielsen,[51] who found 22 per cent Boston Terriers in 50 dogs with mastocytomas, as compared with 4 per cent Boston Terriers in the canine hospital admissions from the Boston area. Larsson,[36] however, found the Boxer to predominate in Sweden, with 29 Boxers among 35 dogs with mastocytomas. No incidence of a primary extracutaneous mastocytoma was found in this study, or in the literature.

The term, "mast cell leukemia," has unfortunately been used for describing basophilic leukemia in cats.[40, 70, 45] However, in none of these 3 feline cases were lesions present in the skin. Also, skin lesions were not present in a dog with basophilic leukemia.[66] It seems that cutaneous mastocytomas and basophilic leukemia are separate neoplastic diseases, with only a histological similarity.

SUMMARY

In the pathological collection studied, mastocytomas comprised 20 per cent of the cutaneous neoplasms of dogs. This tumor rarely occurs in other species. The mastocytomas invaded locally, metastasized, recurred following surgical excision, and were, therefore, classified as true neoplasms. They had a tendency to erosion, grew without demarcation, and had a site preference for the external genitalia (15%) and hindquarters. The Boston Terrier exhibited a marked predisposition, and the Boxer a lesser breed predisposition for this tumor. Males and females were affected with equal frequency. Most mastocytomas occurred after the age of 6 years. Twenty-eight per cent were malignant, 17 per cent recurred between two weeks and one year, and 16 per cent metastasized to inguinal nodes, spleen, liver, and sublumbar nodes. Multiple occurrence was present in 21 per cent.

In no instance did internal mastocytomas occur in the absence of cutaneous tumors. Microscopically, the mastocytoma was characterized by a diffuse proliferation of freely situated round cells of uniform size and shape. With proper stains, a varying number of metachromatic granules were seen in their cytoplasm. Generally speaking, rapidly growing mastocytomas, consisting of immature mast cells, contained only fine dustlike granules, while mature cells had an abundance of coarse granules.

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Experimental Coccidioidomycosis in Dogs

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CANINE COCCIDIOIDOMYCOSIS has been reported with increasing frequency in recent years.[1-4, 6, 8, 11, 13, 14] It must now be considered a disease of common occurrence among dogs in the Southwest. Most diagnoses have been made at necropsy, after progressive spread of the disease led to death or destruction of the subject. Means by which disseminated canine coccidioidal infections can be recognized and confirmed before necropsy have been established [9, 10] and, in one instance, the disease has been proved in a dog that apparently recovered without dissemination.[2] There remains a need for adequate characterization of the primary phases of the disease in the dog. Experimental infection of susceptible dogs would overcome the problem of undetermined date of onset encountered in naturally infected animals.

Coccidioidal infection in man generally occurs as a benign, self-limiting disease (primary coccidioidomycosis or valley fever). In a small percentage of individuals, it may progress as a generalized granulomatous disease to involve any of the body's tissues. About one third of the thoracic radiographs taken early in the course of primary human coccidioidal infection reveal an extensive fan-shaped involvement of a part or the whole lobe of a lung (fig. 1). This lung involvement has always been assumed to be a pneumonia, although associated with remarkably few physical diagnostic signs. The self-limiting nature of the process, with its rapid disappearance, suggests changes other than those observed in bacterial pneumonias.[7] No effort has been made, experimentally or pathologicoanatomically, to determine the exact nature of this particular tissue change. If the dog, following artificial infection with *Coccidioides immitis* spores, developed pulmonary lesions similar to those demonstrated radiographically in about a third of human patients suffering from primary coccidioidomycosis, then serial biopsies of canine lung might serve to clarify the developmental process.

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MATERIALS AND METHODS

Test Animals.—The dogs used were female mongrels, 4 months to 1 year old and weighing 5 to 30 lb. They were housed in an open pen with separate runs, 2 dogs to a run, in an area (on Williams Air Force Base, Ariz.) covered with underbrush and exposed to the climatological conditions under which the average Arizona dog exists. Meat scraps from the mess halls were fed daily.

Thoracic radiographs, complement-fixation and precipitin serological and coccidiodin-senstivity tests, and physical examinations eliminated animals with previous exposure to coccidiodal infection.

Technique of Infection.—The 17 dogs used as test animals were allotted to two groups of 7 and 10 dogs. There was no basis for judging optimal numbers of viable arthrospores to be used in the inoculation, so large numbers were injected in the first group. On the basis of observed response to heavy inoculation, the numbers of spores per dog were greatly reduced for the second group. Of the 7 dogs in the first group, 4 were given 1,000,000 viable coccidioidal arthrospores each and 2 (the youngest and smallest) were given 500,000 spores each. One dog was maintained as an uninoculated control. After work with the first group terminated, the second group was infected—25,000 spores were given to 2 dogs, 10,000 spores to 3 dogs, 1,000 spores to 3 dogs, and 2 dogs were kept as uninoculated controls.

The inoculum consisted of *C. immitis* arthrospores suspended in normal saline solution. Suspensions were prepared by washing old, dry Sabouraud's agar cultures of the fungus, counting the spores in a Neubauer hemocytometer, and checking for viability by standard plate count technique. Dosages were adjusted on the basis of viable arthrospores per cubic centimeter and, depending on the number of spores to be injected, between 1 and 2 cc. of suspension was administered by intratracheal insufflation. A 13-gauge needle was inserted into the trachea between the cricoid cartilage and the first tracheal ring to

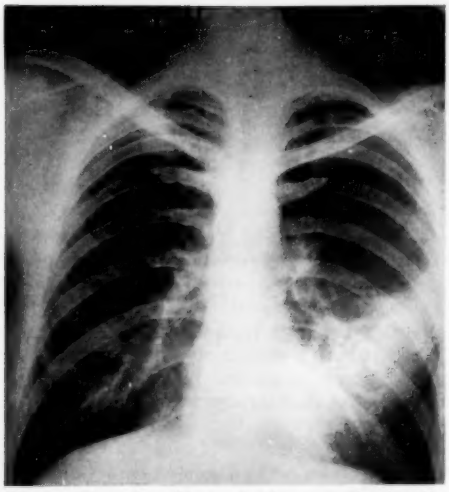


Fig. 1—Thoracic radiograph of a man, showing the fan-shaped infiltrative pulmonary lesion common in primary coccidioidal infection.

serve as a cannula. A 6-inch tomcat catheter was passed through it down the trachea and the syringe, containing the spore suspension backed by a 1 cc. air space between plunger and fluid, was attached. The dog's mouth and nostrils were clamped shut on expiration and released simultaneously with injection of the suspension when there was a strong inspiratory effort.

This technique afforded maximum safety for the attending personnel, while 100 per cent bilateral pulmonary infections were accomplished. A disadvantage to the technique was found to be the development of a coccidioidal abscess in the peritracheal tissue at the injection site. Later, this complication was prevented or minimized by using a longer needle for a cannula and withdrawing the catheter before withdrawing the cannula.

Method of Clinical Observation.—The dogs were observed daily when fed. Rectal temperatures, appetites, appearance of stools, and general attitudes were recorded. At one- to two-week intervals, thoracic radiographs were taken in dorsoventral and lateral positions, and coccidioidin-sensitivity, complement-fixation, and precipitin tests were performed.

Surgical Techniques for Biopsies.—Depending on the radiological appearance of pulmonary tissues, and at various increasing intervals from the time of infection, a certain number of the dogs were subject to thoracotomy with subsequent lobectomy.

The dog to be operated on was anesthetized by intravenous injection of 0.5 per cent pentothal sodium in normal saline solution, given to effect. The dog was then intubated and, by means of a rubber squeeze bag, positive-pressure oxygen ventilation was maintained. This technique facilitated exploration and resection, and provided adequate oxygenation.

Usually, the right thorax was opened with the dog in left lateral recumbency. The incision, in the sixth intercostal space, extended from 3 cm. lateral to the spine ventrally 8 to 10 cm. By means of inspection and palpation, the maximally inflamed lobe was isolated, its blood supply clamped off, and the lobe resected. The vascular and bronchial stumps were then ligated with No. 2-0 chromic catgut and the cavity closed with a continuous heavy cotton suture after negative intrathoracic pressure was restored. Postsurgical bandaging to prevent self-mutilation and wound dehiscence was necessary.

Biopsy and necropsy specimens were fixed in 10 per cent formalin after gross anatomical changes were recorded. Sections prepared from such tissues were stained with hematoxylin and eosin.

Serological and Coccidioidin-Sensitivity Test Procedures.—Tests for precipitins and complement-fixation antibodies were conducted * using the standard technique for both serological procedures.[12] Coccidioidin-sensitivity tests were performed by injecting 0.1 cc. of undiluted coccidioidin intracutaneously on the medial surface of the flank fold. Observation for induration and erythema of the injection site was made 48 hours later.

RESULTS

First Group.—The 2 small, young dogs given 500,000 spores died spontaneously on postinoculation days 9 and 10. Of the dogs given 1,000,000 spores, 2 died immediately after thoracotomy, 1 on day 11, the other on day 13. The 2 remaining dogs inoculated with 1,000,000 spores were killed for necropsy on day 31. One had survived thoracotomy on day 9; no biopsy was taken of the other.

Clinical Observations.—The 6 inoculated dogs in this group became febrile (103 to 105 F.) between postinoculation days 2 and 4, listlessness was manifested between days 5 and 14, and a cough between days 7 and 14. Of the 6, 5 developed diarrhea between days 6 and 21. None of these manifestations were observed in the control dog.

Coccidioidin-Sensitivity and Serological Test Observations.—Of 2 dogs necropsied on day 31, 1 had fixed complement at the 1:32 dilution of serum on day 29. None of the dogs in this group showed a response to intracutaneous coccidioidin.

Radiographic Observations.—The majority of these dogs died before persistent

* By Dr. C. E. Smith, School of Public Health, University of California, Berkeley, with the facilities provided by the Armed Forces Epidemiological Board.

TABLE 1—Complement-Fixation Titers Attained in Dogs Infected with Coccidioides Immitis

Dog (No.)	Onset			Maximum		
	Days after infection	Strength of CF*	Dilution	Days after infection	Strength of CF	Dilution
5	28	4+, 3+, 2+	1:8, 1:16, 1:32	81	4+, +	1:128, 1:256
6	24	4+, 2+, +	1:4, 1:8, 1:16	113	4+, 2+	1:128, 1:256
8	36	4+, 2+, +	1:2, 1:4, 1:8	165	4+	1:128
10	36	4+, 2+	1:4, 1:8	113	4+, +	1:128, 1:256

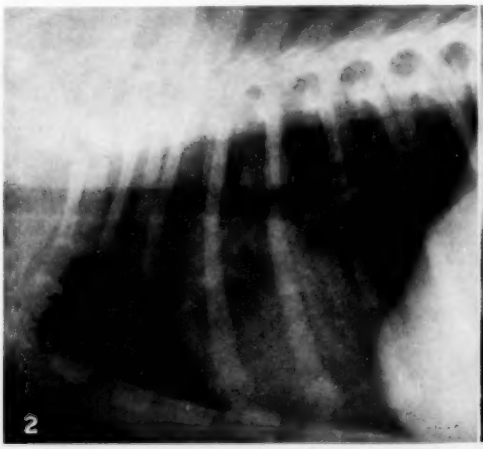
* CF = complement-fixation.

changes could be observed in thoracic radiographs. However, 4 of the 6 dogs revealed some type of change, radiographically, within eight days after inoculation.

Second Group.—Of the 2 dogs infected with 25,000 spores, 1 died on postinoculation day 2 from causes unrelated to the disease. Thoracotomy was performed on 3 dogs on postinoculation days 4, 9, and 11, and necropsies were performed on these dogs on days 9, 10, and 33. The fourth dog undergoing thoracotomy, on day 17, was permitted to survive. The other 3 dogs were not subjected to biopsy and were necropsied on day 185.

Clinical Observations.—Clinical signs shown by dogs in this group were the same as those seen in the first group but were less fulminating. By postinoculation day 7, 3 dogs developed temperatures as high as 104 F.; the remaining 4 had temperatures of 104 to 105 F. by days 21 to 25. Four dogs had no cough, while 3 developed a persistent, harsh cough between the second and fourth weeks. None of these dogs had diarrhea, but all 7 started to show some degree of anorexia and weight loss, marked listlessness, dry mucosae, and loss of hair between the second and sixth weeks. The 4 dogs permitted to survive more than one month followed a declining course for the first 90 days, but gradually regained an appetite with increase in body weight and activity. Five months after infection, all 4 appeared to have a good chance of surviving. The 2 control dogs remained free of the disease.

Coccidioidin-Sensitivity and Serological Test Observations.—In the dogs of both groups, sensitivity to coccidioidin was conspicuously absent for some time. Two lots of the reagent were used on two skin areas in the 4 surviving dogs, but no reactions occurred until after 112 days, when there was a conversion to positive responses. These 4 dogs were the only ones which developed significant complement-fixation antibodies. Precipitins remained absent. All serological tests on the control animals were negative.



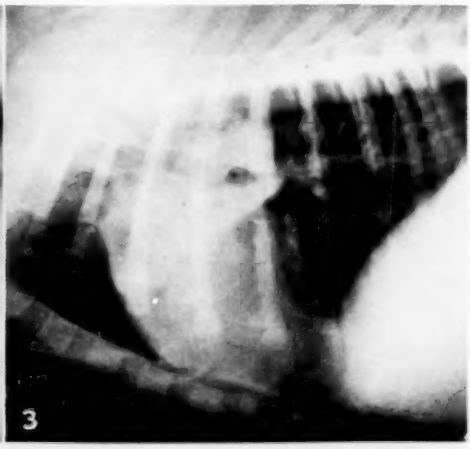


Fig. 2—Lateral thoracic radiograph of dog C, taken before experimental infection with Coccidioides immitis.

Fig. 3—Lateral thoracic radiograph of dog C, taken 13 days after infection. Notice narrowed tracheal lumen and dense shadow at hilus.

Radiographic Observations.—The changes observed radiographically in both groups of dogs can be subdivided into four outstanding patterns.

1) The lumen of the trachea and bronchi was reduced (fig. 3). This was particularly remarkable in 5 dogs of the first and 3 of the second series and was apparently due to pressure of adjacent, inflamed lymph nodes. During each thoracotomy these enlarged nodes were observed.

2) Hilar lymphadenopathy was observed, but with more difficulty. In 1 dog of the first group and 3 of the second group, enlarged bronchial lymph nodes were seen radiographically.

3) Isolated and more-or-less well-circumscribed areas of pulmonary infiltration were seen (often in the form of a small cluster and often peribronchial) in 2 dogs of the first and 4 of the second group. These areas were either localized in one lobe or diffusely spread throughout both lung fields (fig. 5, 7, 8). Several areas seen on radiographs corresponded with coccidioidal granulomas in resected pulmonary tissue.

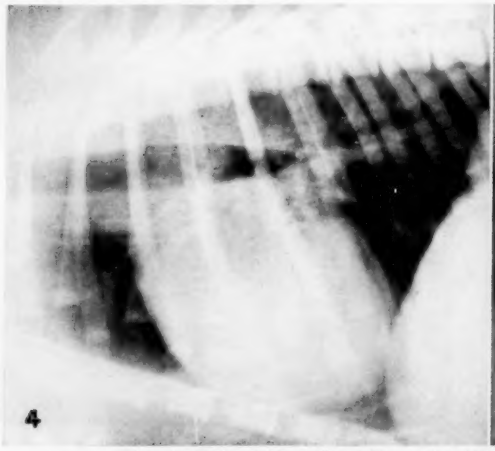
4) The final and least specific pattern, an increase in radiographic density of the bronchovascular pattern, was seen in radiographs taken of 1 dog in the first and 3 of the second group. Occasionally, this process was seen to develop into the nodular peribronchial infiltrate described in the preceding paragraph.

Of the 10 dogs available for serial analysis, based on a series of radiographs taken at intervals following infection, 7 developed lesions observable on radiographs on about postinoculation day 10, while the remaining 3 dogs developed demonstrable lesions between days 14 and 21.

In one half of the dogs, the initial change seen was constriction of the bronchial lumina and, in one third, the first lesions were patchy pulmonary infiltrations. In 80 per cent of the cases, two or more of the listed changes were coexistent after various intervals. Only 2 dogs showed gradual clearing of the changes, observed radiographically, during the first 90 days.

Pathological Changes.—Biopsy or necropsy specimens of pulmonary tissues were available on postinoculation days 4, 7, 9, 10, 11, 13, 17, 31, 33, and 185. The specimens were from dogs inoculated with between 1,000 and 1,000,000 viable coccidioidal arthrospores. Although the clinical course was definitely more dramatic with higher doses, the appearance of lesions was not markedly different.

1) *Gross Findings.*—Significant and uniform findings in all tissue specimens, whether obtained at lobectomy or necropsy, were small, firm peribronchial lesions that extended from the smaller bronchi into surrounding lung parenchyma. In several instances, there were also small, isolated "granulomas" in more peripheral tissue. These increased in number with the pas-



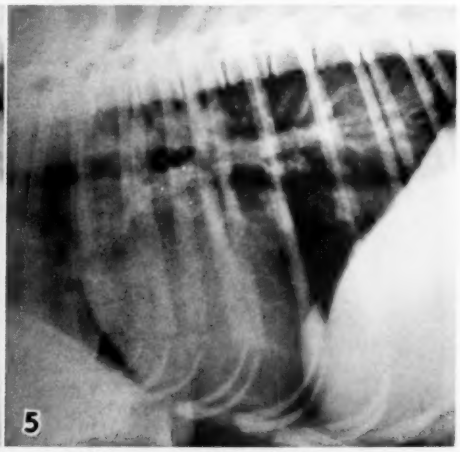


Fig. 4—Lateral thoracic radiograph of dog 10, before experimental infection.
Fig. 5—Lateral thoracic radiograph of dog 10, taken 12 days after experimental infection. Notice nodular peribronchial thickening and increased density of hilar shadow.

sage of time after infection until subpleural lesions became numerous.

Associated with the pulmonary lesions were enlarged, soft, and friable lymph nodes around the main bronchi and in the mediastinum.

With the exception of 2 dogs that showed minute lesions in the parietal pleura, probably the result of direct extension, none of the dogs examined before postinoculation day 33 had lesions in other tissues or organs. This was also the case in 1 of the 3 dogs necropsied on day 185 but, in the other 2 dogs, gross lesions were found in the spleen, kidney, liver, omentum, mediastinal tissue, mediastinal lymph nodes, pericardium, and myocardium. The dog biopsied on day 17 still survives, but a subcutaneous coccidioidal abscess, which formed about day 252, is evidence of dissemination in this animal also.

2) *Microscopic Findings.*—Microscopic observations uniformly confirmed the macroscopic picture. In the earliest specimen, taken four days after inoculation, severe peribronchial inflammation was seen extending into the surrounding alveoli. In this specimen, only a questionable spherule was seen in a mainly polymorphonuclear granulocytic, inflammatory exudate.

In five of the nine specimens obtained from the ninth day onward, the peribronchial tissues were teeming with spherules in various stages of maturation. Some lesions showed small, ringlike structures with amorphous cytoplasm; others showed matured, frequently ruptured spherules that expelled endospores into surrounding tissue.

Alveoli in a majority of the sections contained an exudate mainly of polymorphonuclear granulocytes, with some macrophages. Both solitary and multiple granulomatous processes were seen in some alveoli. These true granulomas consisted of large epithelioid cells surrounding a granulocytic, necrotic exudate, often with a spherule in the center.

Apparently, the infection spread via the lymphatics to peribronchiolar and peribronchial tissue. Sections of associated regional lymph nodes, available from 4 of 9 dogs sampled, showed profuse multiplication of coccidioidal spherules as early as the ninth day, supporting the assumption of rapid lymphangitic spread.

Discussion

It is evident from the observations that primary pulmonary coccidioidomycosis in the dog presents no significant, pathognomonic clinical syndrome. Fever, cough, listlessness, and weight loss over a prolonged period are relatively nonspecific, yet these manifestations should suggest coccidioidomycosis as one possible cause of the respiratory affliction observed if the dog lives, or has lived, in the enzootic area.

The clinician may be aided in differentiation by the following procedures:

1) By taking serial radiographs in dorsoventral and lateral positions which, in ten to 14 days, may show typical, though not pathognomonic, changes sufficient to suggest a diagnosis of coccidioidomycosis.

2) By submitting serum for complement-fixation tests after the first month from onset of signs; antibodies will usually develop and will confirm the diagnosis.

3) By testing for coccidioidin sensitivity; though admittedly occurring late in this group of dogs, this should also aid in differential diagnosis.

The spread of arthrospores after deposition in the trachea and bronchi, as indicated by section of serial biopsy and necropsy specimens, was apparently to the smaller bronchioles and into the alveoli. With a particle size of about 3 μ, it can be assumed that arthrospores reached the alveoli proper. There the spores apparently change from vegetative to parasitic form, as spherules of various sizes and stages of maturation were observed in alveoli as early as the ninth day after infection.[16,17] Subsequently, the spread of the parasitic phase appears to be either by direct extension to neighboring alveoli, moving peripherally toward the pleura, or by the lymphatic system toward the regional lymph nodes, establishing peribronchial and peribronchiolar inflammation along the way.

Although this series of animals was too small to determine the successive response of host to mycelial elements and spores, or to spherules, it appeared that the early polymorphonuclear granulocytic exudate and the subsequent mononuclear response forming the typical granuloma are in keeping with Forbus'[5] findings in man, as well as with Tarbet's[17] observations in the mouse. The predominance of the polymorphonuclear granulocytic reaction could be explained either by the high number of spores injected or by a peculiar slow immunogenic response (possibly related to

late onset of skin test conversion) in the dog.

The exact nature of the peculiar fan-shaped infiltrative lesions, observed in 30 per cent of all primary pulmonary cases in man, was not further clarified by the radiographic and pathological appearances of pulmonary changes in the dog. That this manifestation in man is an interstitial hypergic reaction,[7] spreading along the vascular channels or lymphatics toward the pleura peripherally to the actual focus, could not be confirmed in the canine test animal.

On the contrary, the typical radiographic patterns described in the remaining approximately 70 per cent of human primary cases (slight increase in hilar density, lobular and sublobular patchy exudates and infiltrates, peribronchial "bronchopneumonic" infiltrates, and hilar lymph node enlargement[7,15]) seem to correspond well with the radiographic observations in the dog ("Radiographic Observations," paragraphs 2, 3, 4).

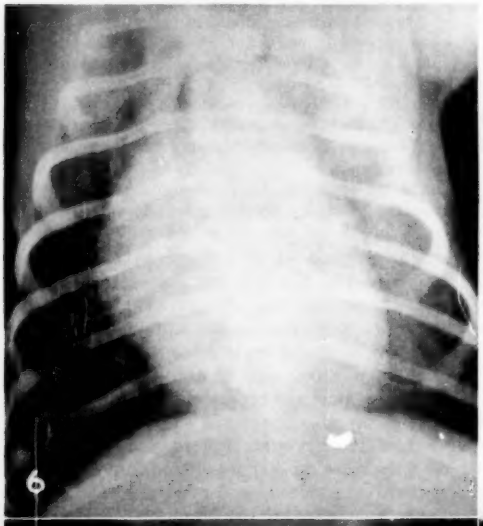
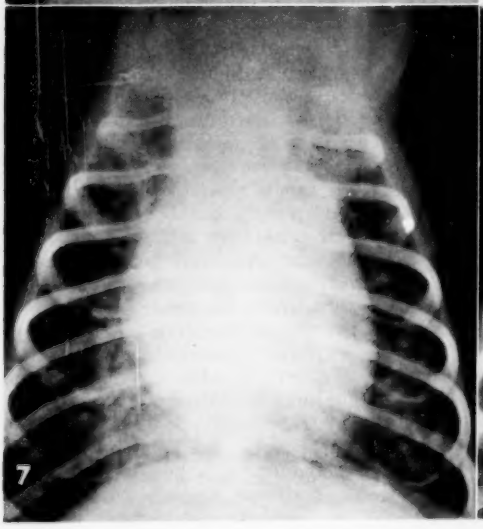
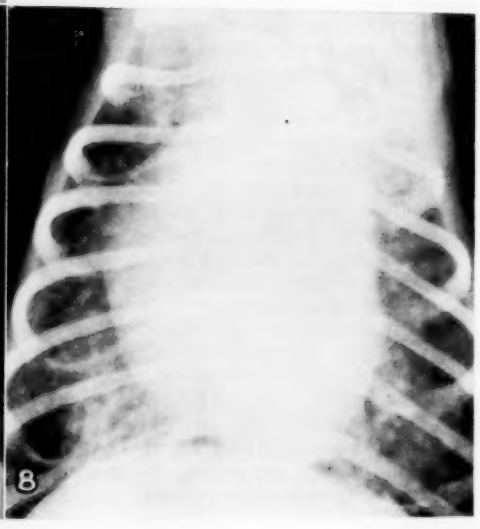


Fig. 6—Dorsoventral thoracic radiograph of dog 5, before experimental infection.

Fig. 7—Dorsoventral thoracic radiograph of dog 5, taken 30 days after experimental infection. Notice diffuse miliary nodules in both lung fields.

Fig. 8—Dorsoventral thoracic radiograph of dog 5, taken 51 days after experimental infection. Notice confluent nodular lesions in both lung fields.





While this series is small, it has provided information of considerable background value for further work now under way. Dogs of the age group used are apparently good test animals, regardless of breed. From their responses to 1,000 spores, that number or fewer seem to be sufficient to infect. There will be considerable error in dosage when numbers of viable spores per animal are reduced below 1,000 by dilution techniques. The described technique of intratracheal insufflation with a suspension of arthrospores was an effective method of inoculation, mimicking natural exposure as closely as possible, with minimal exposure to attending personnel. However, it is suggested that workers not previously exposed to natural sources of coccidioidomycosis avoid such exercise.

SUMMARY

Responses of dogs to infection by intratracheal insufflation with *Coccidioides immitis* arthrospores were observed to determine pathogenesis of the canine disease in its early stages and to evaluate the usefulness of the dog as an experimental animal for the study of coccidioidomycosis in man.

Signs of cough, listlessness, diarrhea, anorexia, weight loss, dry mucosae, and loss of hair appeared in two to 28 days after infection. Dogs infected with doses of 500,000 or 1,000,000 spores each became sick earlier and experienced a more severe illness than those infected with 1,000, 10,000, or 25,000 spores each.

Gross and microscopic examination of tissues obtained by biopsy or necropsy at intervals from four to 185 days after infection revealed changes ranging from inflammation, in earliest lung tissues taken, through granuloma formation and dissemination from thoracic to abdominal viscera, in later samples. Changes similar to those seen in about 70 per cent of thoracic radiographs taken of human patients with primary coccidioidomycosis developed in the dogs in between eight and 21 days. A fan-shaped infiltrative pulmonary lesion peculiar to approximately 30 per cent of primary coccidioidomycosis in man was not observed in thoracic radiographs of the dogs.

Complement-fixing antibody appeared within 28 to 36 days, but precipitins were not detected in serums from the experimentally infected animals. Skin sensitivity to coccidioidin was not demonstrated before postinoculation day 112.

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Symposium on Chronic Respiratory Diseases of Poultry

I. Isolation and Identification of Pleuropneumonia-like Organisms of Avian Origin

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CHRONIC RESPIRATORY DISEASE (CRD) has been known for a number of years. In 1943, Delaplane and Stuart [5] suggested the name and the theory that the disease was caused by a virus. In 1952, a group of investigators [4] concluded that CRD was caused by the agent described previously by Delaplane.

Markham and Wong [13] succeeded in isolating pleuropneumonia-like organisms (PPLO) from exudates of chickens and turkeys with CRD. The cocco-bacilliform bodies described by Nelson almost 20 years earlier were probably identical with this agent.[13] Nelson,[14] in 1936, used chicken inoculation to isolate and identify this infection. He grew the agent in chicken embryos and in tissue culture [15] and, three years later,[16] succeeded in growing it on serum-enriched, cell-free mediums.

Delaplane [6] had produced a CRD in chickens and sinusitis in turkeys with a so-called "viral" agent cultured in chicken embryos. In 1952, Van Roekel *et al.*[21] used all the cultural procedures, except the tissue culture method described originally by Nelson, to study CRD. Following the observation that PPLO were associated with CRD,[13] other artificial culture methods were developed. Grumbles *et al.*[9] described a medium composed of a fermentable carbohydrate and a phenol red broth base enriched with bovine serum fraction. This medium was simpler than that of Markham and Wong, which contained beef heart infusion and a yeast extract enriched with equine serum. Adler *et al.*[1] developed a more complicated medium, which seemed satisfactory for the growth of PPLO of avian origin. The medium had, in addition to the substances described, a whole blood agar slope. Other mediums and methods for the isolation of PPLO have been described by Lecce and Sperling,[12] Taylor *et al.*,[19] and Hofstad and Doerr.[10]

The identification of CRD was first based upon finding typical gross pathological changes in the turkeys or chickens. Jungherr *et al.*[11] used microscopic tissue changes to differentiate CRD from Newcastle disease, infectious bronchitis, and laryngotracheitis. As soon as PPLO were found to be capable of producing this disease, diagnosis of the infection was based on the chicken embryo death pattern or on isolation of PPLO on culture mediums. The first serological test to be used was the hemagglutination-inhibition test, originally observed and used by Van Herick and Eaton,[20] followed by the use of the tube-agglutination, slide-agglutination, and whole blood-agglutination tests. These tests indicated that the PPLO of avian origin were antigenically related.[2, 22]

By the time this study was started, a procedure (fig. 1) for isolation and identification of PPLO had been developed at the University of California.

In a previous study,[2] two strains of PPLO, isolated from the infraorbital sinus of a turkey with infectious sinusitis, could be separated by their differences in susceptibility to streptomycin. One was readily propagated on artificial mediums and was not pathogenic for turkeys and chickens; the other did not readily colonize on serum-enriched agar and was capable of producing disease.

Additional study with cross-agglutination tests demonstrated the presence of at least three serologically different strains of PPLO.[2] Strains which failed to produce respiratory tract lesions were not related antigenically to the S_6 strain which is used for standard antigen production at the University of California and which is related antigenically to all of the "pathogenic" strains of PPLO isolated at this laboratory or received from other laboratories up to this time. Some metabolic differences between strains were noticed. The strains that did not produce lesions in turkeys were designated as "nonpathogenic". They were more active in fermenting sucrose and their growth on PPLO-serum agar was more rapid than the standard, lesion-producing pathogenic strain (S_6).

To evaluate the significance of these strain differences, a series of PPLO isolations was made from groups of apparently normal and diseased birds. The organisms were characterized by the different methods described in this report.

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MATERIALS AND METHODS

The method for acquiring and typing the various strains of PPLO are essentially as described previously.[2] The mediums (blood slope overlay and PPLO agar) used in these studies were as described,[1] except for the addition of 1 per cent yeast hydrolysate * to the agar and enrichment broth. The rates of growth of the various PPLO were determined by the rapidity with which visible colonies could be detected on serum agar plates with a dissecting microscope at 108 diameters. The rate of growth was considered rapid if colonies could be observed within 24 hours, and slow if colonies were visible only after 72 hours. The maximum colony size attained by the slower growing strains was 0.1 mm. in diameter. Colonies of the rapidly growing strains were frequently 0.4 mm. or more in diameter within 48 hours (fig. 2).

The following technique is used to study the morphology of PPLO in liquid mediums: (1) Prepare a smear and gently heat-fix it by flaming the slide. (2) Flood the slide with Bouin's solution and allow it to fix for one half hour. (3) Wash the slide thoroughly in tap water. (4) Stain by flooding the slide with Giemsa's stain, composed of 1 drop of stock solution per milliliter of distilled water, buffered at pH 7.0, and allow it to act for 30 minutes. The stain is freshly prepared for each use.

Using Dienes' method,[7] colonies that appear to have the general characteristics of PPLO are cut from the plate and placed, colony side up, on a slide and a stained cover slip is placed, stain side down, on the colonies. The stain used is described by Dienes[7] as containing 2.5 Gm. of methylene blue, 1.25 Gm. of azure II, 10.0 Gm. of maltose, 0.25 Gm. of Na_2CO_3, 0.2 Gm. of benzoic acid, and 100.0 ml. of distilled water.

With a cotton swab, the stain is applied in a thin film over the surface of the cover slip and allowed to dry. The stain on the cover glass is not affected by storage for at least two months, if stored in a Petri dish or another covered container.

Several minutes should be allowed for the colonies to take up the blue stain from the cover slip. The stained colonies are examined at 100, 500, and 1,000 diameters. Colonies of PPLO will contain small coccoid forms (about 0.25 to 0.5 μ) in the fine lacey peripheral portion of the colony. Larger coccoid and rod forms are never seen in PPLO colonies. Other bacterial colonies, with the exception of *Hemophilus gallinarum*, usually decolorize the stain. Colonies of the type that do not give typical reactions should be cut out of the agar and rubbed over a new serum agar plate.

Additional procedures will be described as they were used in the course of the experiment.

RESULTS

Isolation and Characterization of PPLO

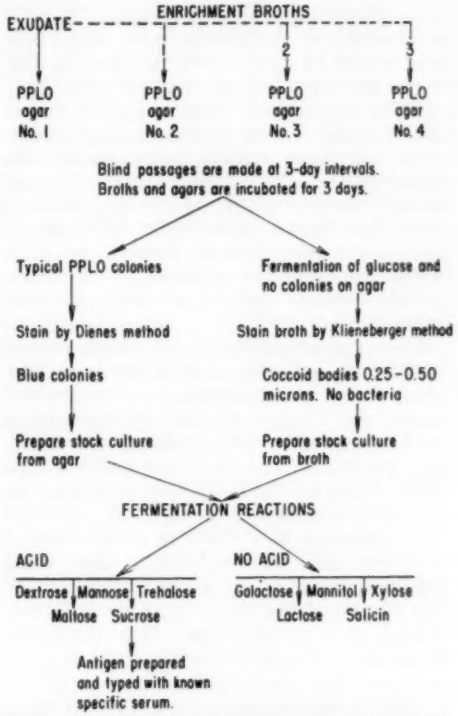

Fig. 1—Procedure for the detection and identification of pathogenic pleuropneumonia-like organisms (the composition of the mediums used is described in "Materials and Methods").

Strains from Birds Free of Clinical Signs of Disease.—Isolations of PPLO were made by tracheal swabs from a closed flock of chickens belonging to the University of California. This flock had been studied for serological reactions with the S_6 antigen for three years. One month before sampling, 32 per cent were reactors.

The second flock used was a commercial chicken-breeding flock that had been on a PPLO egg-transmission control program for approximately three years. This flock was composed of two groups of birds, 12 and 22 weeks old, that were serologically negative, and one group of 10-month-old birds that had agglutinins for the S_6 antigen. All the birds were clinically free of disease.

Tracheal swabs were taken from 50 birds of the University flock and PPLO were recovered from 49. In the hatchery flocks, PPLO were not recovered from the tracheal swabs taken from 25 birds in each of the

* Nutritional Biochemicals Corp., Cleveland, Ohio.

two serologically negative groups. Pleuropneumonia-like organisms were recovered from ten of 11 swabs from the third group at the hatchery, 80 per cent of which reacted to the agglutination test. The large-sized, rapidly growing colonies on cultures of all of these isolates were similar in appearance to the nonpathogenic type. Stained preparations of these cultures, which had been incubated for 72 hours or longer, showed a large number of ring forms, while similar preparations of pathogenic strains contained only coccoid forms.

Five typical cultures were selected from each of these same sources for more detailed study. Two nonpathogenic partridge strains and three virulent turkey strains were included in these studies for reference purposes. All the PPLO isolated fulfilled Sabin's [17] criteria for inclusion in the pleuropneumonia group. All cultures were passaged five times in noninhibitory broth to test the possibility that these organisms were L variants of a bacterium. The results of the characterization of these strains are shown (table 1). Although these nonpathogenic isolates grew rapidly and produced large colonies, nine of the 12 strains failed to ferment glucose, maltose, sucrose, mannitol, and lactose. All were agglutinated by rabbit antiserum prepared against a known nonpathogenic type. None of the antigens were agglutinated by the standard pathogenic (S_6) antiserum. The ammonium sulfate flocculation levels were the same as those found previously [2] with the nonpathogenic types.

Each of the strains was propagated in embryonating chicken eggs from a source that was free of PPLO. Yolk harvested from embryos inoculated with each strain was inoculated into the abdominal air sacs and infraorbital sinuses of 3 turkey poults, 6 weeks old. Blood samples taken from the poults at the same time were negative for agglutinins of all the strains studied. Several additional birds were inoculated with normal yolk as controls. Four weeks after exposure, blood samples were taken and all poults were necropsied.

Two serologically negative chickens were inoculated intraocularly with a fifth passage culture of each of the strains that did not produce respiratory lesions. Two virulent strains were included in this trial as positive controls. Sterile broth was inoculated into the anterior chamber of the eye as a negative control.

Although the cultural and serological examinations of the chicken and the partridge strains indicated that they belonged to the nonpathogenic group, the data (table 1) show that some lesions were found in the air sacs of poults inoculated with six of these 12 strains. No lesions

TABLE 1—Characteristics of Some Representative Strains of Pleuropneumonia-like Organisms of Avian Origin

Strain	Rate of growth	Fermentation of carbohydrates	Antigen reaction to type of antiserum*	$(NH_4)_2SO_4$ flocculation level (%)†	Turkey inoculation**			Chicken eye inoculation‡	
					Lesions		Serological reaction		
					Air sac	Sinus		Bird 1	Bird 2
Chicken									
1	Rapid	+	C	24	1 S	0	1 S_6	ND§	ND
2	Rapid	+	C	24	1 S	0	1 S_6	ND	ND
3	Rapid	—	C	24	0	0	0	2+	2+
4	Rapid	—	C	24	0	0	0	1+	2+
5	Rapid	—	C	24	0	0	0	—	2+
6	Rapid	—	C	24	1 S	0	1 S_6	ND	ND
7	Rapid	—	C	24	0	0	0	1+	2+
8	Rapid	—	C	24	0	0	1 S_6	3+	—
9	Rapid	—	C	24	0	0	0	—	2+
10	Rapid	—	C	24	1 S	0	1 S_6	ND	ND
Partridge									
1	Rapid	—	C	24	1 S	0	1 S_6	ND	ND
2	Rapid	+	C	24	2 S	0	2 S_6	ND	ND
Turkey‖									
1	Slow	+	S_6	16	3 M	2 M	2 S_6	3+	+
2	Slow	+	S_6	16	3 M	3 M	1 S_6	ND	ND
3	Slow	+	S_6	16	3 M	2 M	2 S_6	3+	3+

* All antigens were tested against S_6, C, and N antiserums; † percentage concentration of $(NH_4)_2SO_4$ that will flocculate the PPLO antigen; ** each strain was inoculated into the sinuses and air sacs of 3 poults. Blood samples were taken and all poults were necropsied four weeks later. Lesions were graded as "S" for slight, "M" for marked, and "0" for none. Serums were tested against all three (S_6, C, and N) antigens. The number beside the letter indicates the number of poults with serological reactions or showing lesions. ‡ Eye lesions are graded as mild (+), moderate (2+), and severe (3+); § ND = not done; ‖ the three turkey strains are pathogenic.

were produced in turkeys inoculated with the remaining six strains. In each instance, the air sac lesions were mild, consisting of numerous localized areas of thickening usually not more than 2 mm. in diameter. None of these birds had sinusitis or tracheitis. This was in marked contrast to the lesions produced in turkeys inoculated with the pathogenic strains, which had severe and extensive aerosacculitis, as well as sinusitis and tracheitis. The groups of birds that had lesions after being inoculated with nonpathogenic strains (chicken strains 1, 2, 6, and 10, and partridge strains 1 and 2) also had agglutinins for the standard antigen type (S_6) at necropsy. However, no agglutinins were present for homologous strains.

The six strains that failed to produce tracheal and air sac lesions (table 1) were capable of inducing lesions of the eye. These lesions were graded as mild (+), moderate (2+), and severe (3+). The mild lesions were localized areas of lymphocytic infiltration without heterophilic reaction; a 2+ lesion was more extensive and accompanied by a heterophilic reaction; and a 3+ lesion was extensive and marked by a severe heterophilic response. In all instances, the ciliary body was the most consistently affected area. Lesions graded 2+ and 3+ could be detected grossly as well as microscopically.

Isolation of PPLO Strains from Birds with Clinical CRD.—An additional 84 samples of tracheal exudate were examined from chickens and turkeys with field cases of CRD. Examination of the colony types of the organisms isolated from these cases revealed a predominance of the nonpathogenic type of PPLO. Only five of the 84 exudates cultured yielded pathogenic PPLO. The organisms were characterized on the basis of growth and antigenic properties. Antigens prepared from this group of nonpathogenic strains also were agglutinated by hyperimmune rabbit serum for the nonpathogenic type of PPLO but not for the pathogenic type. Many of the strains failed to ferment any of the carbohydrates. Since most of these isolations were made from field cases of CRD, the failure to isolate a larger percentage of pathogenic types of PPLO was of considerable concern.

Isolation and Characterization of PPLO Isolated from Day-Old Poults.—An inves-

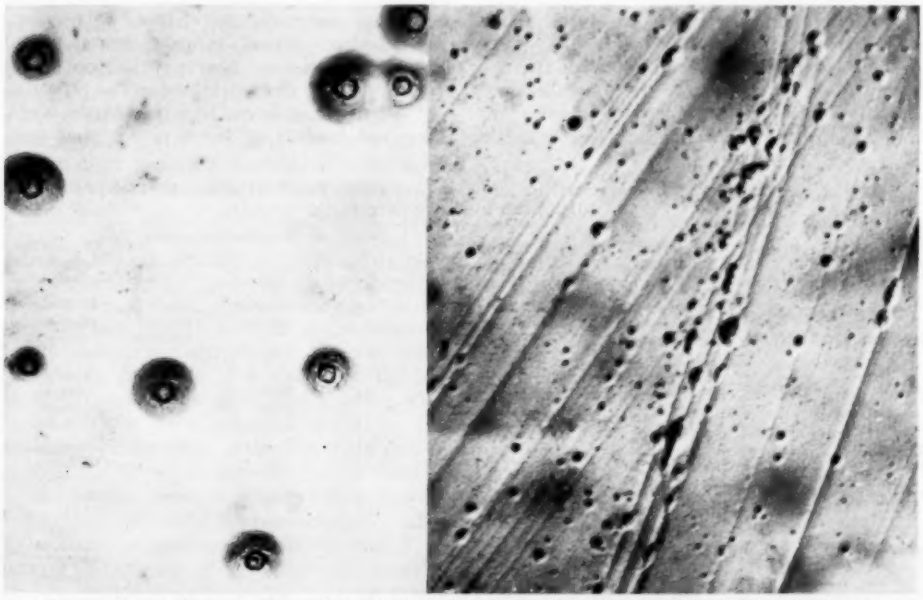

Fig. 2—Comparison of colony size of avian pleuropneumonia-like organisms. The colonies on the right are slow-growing pathogenic type colonies after 72 hours of incubation at 37 C. on serum agar plates. The colonies on the left are rapidly growing types under similar conditions. x 100.

TABLE 2—Occurrence of Air Sac Lesions and Isolations of Pleuropneumonia-like Organisms from Day-Old and 3-Week-Old Poults

	Day-old poults				3-week-old poults			
Group	No.	No. with air sac lesions	Passive agglutinins S_6	PPLO isolations	No.	No. with air sac lesions	Passive agglutinins S_6	PPLO isolations
A	11	5	—	—	3	3	—	+N
C	20	7	—	+N*	3	2	—	+N
H	12	3	—	—	3	2	—	+N
G	12	4	+	+N	3	3	—	—
OB	11	5	—	+S_6	ND†	ND	ND	ND
OW	7	0	—	—	2	2	—	—
R	23	1	—	+N	3	2	—	+N
WL	19	3	—	+N	1	1	—	—
WS	23	3	—	+N	3	1	—	+N
WW	25	3	—	+N	ND	ND	ND	ND

* +N: the PPLO isolated were *N* type; † ND = not done—this flock was destroyed.

tigation was made of the type of organisms that would be found in the egg-transmitted disease in poults.

A large sampling of day-old poults was made from ten breeding flocks from four states. From each flock, culled poults were necropsied at 1 day and at 3 weeks of age. In birds from all ten flocks, there were air sac lesions, usually in the thoracic air sacs. These lesions consisted of masses of yellow caseous exudate and varied from barely detectable foci to masses involving the entire air sacs.

Cultures from the poults with aerosacculitis were made on the blood slope overlay medium, and PPLO were isolated from poults from nine of the ten flocks. The incidence of lesions correlated with the recovery of PPLO. Data on these strains are shown (table 2). Serological typing of these organisms showed that eight strains were antigenically related to each other and did not have antigenic components in common with any of the strains studied previously. The new antigenic type was designated "N" for the purpose of identification. The ninth strain was antigenically identical to the standard pathogenic (S_6) strain.

The observation that a new antigenic type of PPLO was egg-transmitted from many flocks led to a reinvestigation of a strain of PPLO isolated 18 months previously under similar circumstances. At that time, 67 culled poults, 1 day old, were examined for air sac lesions as part of a screening program to detect PPLO egg-transmission. Blood samples taken from these poults before necropsy showed that 25 had passive agglutinins for the S_6 antigen. Pleuropneumonia-like organisms were recovered from 8 of the 9 poults that had

TABLE 3—Summary of Characteristics of Five Antigenic Types of Pleuropneumonia-like Organisms of Avian Origin

Type	Antigenicity*	Cultural characteristics	Carbohydrate fermentation	$(NH_4)_2SO_4$ ppt.** (%)	Turkey inoculation lesions		Chicken eye inoculation lesions
					Air sac	Sinus	
Standard antigenic and pathogenic, S_6	S_6	Slow growing; some strains failed to grow on artificial mediums.	All strains that have been adapted will ferment.	16	Marked reaction.	Slight to marked reaction.	Marked reaction.
Typical nonpathogenic, C	C	Rapid growing.	Some strains ferment, many do not.	24	None, or† slight reaction.	None.	None, to marked reaction.
Single isolation, SA	SA	Rapid growing.	Will ferment.	24	None.	None.	None, to slight reaction.
Single isolation, O	O	Rapid growing.	Does not ferment.	24	None.	None.	None, to slight reaction.
Egg-transmitted strain from poults, *N*	N	Rapid growing; seems to grow better in the presence of agar.	Does not ferment.	†	Moderate reaction.	None.	None, to slight reaction.

* Each strain reacted only with its homologous antiserum, and there was no degree of cross reaction between types of PPLO; † the significance of this finding is reviewed (Discussion section); ** the amount of $(NH_4)_2SO_4$ required to precipitate the organisms.

aerosacculitis. When 50 additional poults from the same flock were placed in isolation and necropsied four weeks later, 6 also had aerosacculitis. Pleuropneumonia-like organisms were recovered from the 6 birds, but none of the 50 poults had agglutinins for the S_6 antigen. When another 50 poults from the same flock were necropsied at 9 weeks of age, 9 had air sac lesions, and PPLO were recovered from 8 of these 9 birds. The agglutination test on all 50 birds was negative for the standard antigen. Additional blood samples from 300 birds in this flock were also negative for S_6 agglutinins.

The PPLO strain isolated 18 months earlier was compared with the newly isolated antigenic type and was found to be an N type. The growth characteristics and fermentation patterns of all of the N type strains were identical. A comparison of the characteristics of the five antigenic types of PPLO isolated from birds up to this time shows the serological, biochemical, and pathological differences in PPLO of avian origin (table 3).

DISCUSSION

In a series of attempts to isolate and characterize PPLO from the tracheas of birds with varied histories, no PPLO were isolated from 50 birds that were serologically negative to the standard antigenic strain and free of clinical signs. These results were consistent with previous experiments. However, PPLO were isolated consistently from 61 birds that were serologically positive to the standard antigenic strain and free of clinical signs. When characterized, all of these isolated PPLO were of the nonpathogenic type. The consistent isolation of only the nonpathogenic type of PPLO from birds with agglutinins for the standard antigenic (pathogenic type) strain was surprising. It was more surprising that the PPLO subsequently isolated from the tracheas of 84 birds with signs of CRD were predominantly of the nonpathogenic type; only five were of the pathogenic type. A representative number of the nonpathogenic strains were characterized by several different means (table 1). These organisms were identical in morphology, rate of growth, antigenicity, and ammonium sulfate flocculation levels to the nonpathogenic type described previously;[2] however, variations were observed in carbohydrate fermentation. Some of the strains produced acid in maltose, dextrose, and sucrose, but not in lactose and mannitol. This was the typical fermentation pattern for avian PPLO,[9] which was observed previously in both pathogenic and nonpathogenic strains.[2] However, nine of these nonpathogenic strains failed to produce acid in any of the carbohydrates tested, even when incubated for 14 days. The significance of this finding will be discussed.

These nonpathogenic strains also were tested for pathogenicity by bird inoculation. The basic criterion for pathogenicity in this study was the production of gross lesions in the respiratory tracts (air sacs, lungs, sinuses) of turkeys.

The data (table 1) indicate that some of the nonpathogenic strains produced slight air sac lesions. There was no correlation between the air sac lesions of poults and those found in the eyes of chickens after intraocular inoculation with the same strains. Strains that failed to produce respiratory lesions in turkeys were able to produce eye lesions in chickens.

It was significant that all groups of turkeys which developed air sac lesions following inoculation also developed agglutinins for the pathogenic strain. None of the groups exposed to the nonpathogenic type developed homologous agglutinins. The unexpected development of lesions and antibodies may have happened because the inoculum contained some pathogenic types in spite of careful attempts to select only nonpathogenic colonial types. It was possible that the selected agar block cutouts contained minute colonies of the pathogenic type; our criteria of colony type may not be adequate to differentiate pathogenic from nonpathogenic types.

The significance of the presence of nonpathogenic types in birds with or without clinical signs of disease is not clear. Apparently the nonpathogenic strains are widely distributed in birds and can be confusing in the determination of the role of PPLO in CRD.

The isolation of large numbers of nonpathogenic and relatively few pathogenic types from birds with clinical CRD might be due to the presence of mixtures of these types in the tissues. The isolation of only nonpathogenic types from birds with S_6 agglutinins but without clinical signs of disease might be explained similarly; only

nonpathogenic types may have persisted after clinical recovery. Also, the nonpathogenic types may have adapted to artificial mediums more readily.

Some pathogenic PPLO did not grow readily in cell-free culture mediums. Some, which were isolated only after many blind passages in mediums, were capable of producing lesions in poults by air sac inoculation, with the eventual production of agglutinins for the standard pathogenic type. However, these strains were erratic in their ability to produce colonies on PPLO agar. Thus, the adequacy of the PPLO isolation procedure proposed by Leece *et al.*[12] becomes doubtful.

The occurrence of PPLO strains that do not ferment carbohydrates (tables 1, 3) presents a problem in the use of isolation techniques that utilize this characteristic, such as those described by Grumbles *et al.*[9] and Taylor *et al.*[19] The possibility of mixtures of types of PPLO also presents difficulties in the use of the isolation technique described by Adler *et al.*[1]

An opportunity arose to examine, for PPLO infection, a large number of day-old poults from ten sources in four states (table 2) and aerosacculitis was found in all flocks. Birds from all but one flock were found to be infected with a new antigenic type of PPLO (type N). An additional four strains, from other groups of poults under similar circumstances, were antigenically related to this new type. Apparently the N strain is widely distributed in poults and is readily egg transmitted.

Further examinations of poults from these groups at 3 and 8 weeks of age indicated that well-defined air sac lesions persisted through this period. Only N-type PPLO were isolated from these lesions, and the serums of these birds did not contain agglutinins for the standard antigenic type; the sinus lesions and tracheal râles usually seen in infections with the standard antigenic type also were absent. The course of infection with the N type of PPLO and its clinical and epizoological significance are being studied. Whether this disease would persist throughout the bird's life and, if so, what its significance would be are not known.

The recognition of N-type infections in turkeys makes it necessary to re-evaluate some of our present concepts of PPLO infection. For example, turkeys may develop severe air sac infections without manifesting detectable signs of disease. Therefore, sinusitis is not a good criterion for the evaluation of the presence of PPLO infection in turkeys.

Also, since turkeys with N-type PPLO did not develop agglutinins to the standard antigenic (S_6) type, any testing program in turkeys dependent on this single antigen type will not be entirely satisfactory in the prevention of egg-transmitted PPLO infection. Whether a similar problem exists in chickens has not been determined.

At the present time, all cultural and serological procedures used for the diagnosis of PPLO infection need re-evaluation. The findings described in this paper are, in many instances, preliminary in nature and care should be used in their interpretation. Future studies with avian PPLO must consider the significance of antigenic, cultural, and pathogenic variations in this group of organisms.

SUMMARY AND CONCLUSIONS

The literature on the isolation and identification of pleuropneumonia-like organisms (PPLO) of avian origin is reviewed briefly, and the techniques used at the University of California are given in detail. The occurrence of serological and pathological variations found in earlier investigations are described.

Pleuropneumonia-like organisms that did not produce disease in birds were observed. They were not related serologically to the standard pathogenic strain (S_6) which was isolated from turkeys and which has been widely used for antigen production. Some strains of the pathogenic type failed to grow on agar.

A number of PPLO isolated from newly hatched poults with aerosacculitis did not ferment carbohydrates. These isolations were of one type and were not related antigenically to the standard pathogenic strain.

Since PPLO with antigenic, physiological, and pathogenic characteristics differing from the standard pathogenic strain have been isolated from chickens and turkeys, it is evident that procedures for the detection of PPLO infections in poultry should be re-evaluated, and that the pathogenicity for birds of PPLO isolated from avian tissues must be determined.

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Symposium on Chronic Respiratory Diseases of Poultry
II. The Role of *Escherichia Coli* in the Cause of Chronic Respiratory Disease and Certain Other Respiratory Diseases

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CERTAIN BACTERIA and viruses[1,4,9] can increase the severity of chronic respiratory disease (CRD). Chronic respiratory disease complicated with *Escherichia coli* is characterized by fibrinous pericarditis, perihepatitis, aerosacculitis and, at times, salpingitis and panophthalmitis.[4-6]

Chickens or turkeys, 3 to 16 weeks old, artificially infected with *Esch. coli* were depressed, showed anorexia, and had a body temperature of 108 to 110 F. or lower during terminal stages. In acute infections, there was often a yellowish diarrhea. There was a mononuclear and heterophilic infiltration of the pericardial sac, air sacs, lung, and choroid.

Groups *b*, *c*, and *d* have been typed by the Communicable Disease Center at Chamblee, Ga. Type *b* belongs to O group 78 and has an "A" or capsular surface antigen; type *c* belongs to O group 2 and has the "L" envelope antigen No. 1; and type *d* belongs to O group 1 and also has No. 1, L antigen. These same types have been recovered from cases of peritonitis without CRD, and the lesions have been reproduced by inoculating these types into the oviducts of laying birds.

While pericarditis could be produced by inoculating broth cultures of these pathogenic types of *Esch. coli* into the air sacs, the incidence and mortality could be increased by adding pleuropneumonia-like organisms (PPLO), India ink,[4] infectious bronchitis virus (IBV), or Crawley virus to the inoculum.

TABLE 1—Isolations of Various Types of Escherichia Coli and Their Percarditis-Producing Ability

	Types of *Esch. coli*						
	2–1–5 *	*a*	*b*	*c*	*d*	*e*	*f*
No. of isolations	1	1	10	19	6	1	2
Pericarditis	3/10†	1/10	3/10	8/10	5/10	0/10	2/10

* Culture obtained from the Communicable Disease Center of the U.S. Public Health Service (2–1–5 = #2 "O", #1 "K", and #5 "H" antigens); † numerator = number of birds developing pericarditis, and the denominator = number inoculated into the lesser abdominal air sac with a 24-hour tryptose broth culture of the type indicated.

MATERIALS

From each of 2 turkeys and 38 chickens, an isolate of *Esch. Coli* was recovered from the pericardial sac. These isolates could be placed in six serological groups *(a, b, c, d,* and *f)* on the basis of their "O" antigens. The number of isolations of each serological group, together with the number of 5-week-old chickens developing pericarditis after air sac inoculation of a 24-hour tryptose broth culture, are shown (table 1).

The chickens used in these experiments were obtained from the Poultry Department of the Virginia Agricultural Experiment Station or from our own flock of PPLO-negative layers. The turkeys were from our own PPLO-negative flock. During these experiments, chicks and poults maintained negative PPLO serum-plate agglutination reactions unless they were inoculated with PPLO. These birds could not be shown to be naturally infected with any disease-producing organisms. Birds of both sexes were used between 3 weeks and 6 months of age. All birds were kept in isolation rooms, each of which had its own air supply. All groups of birds exposed to IBV, Crawley virus, or to aerosols of *Esch. coli* were maintained in separate modified Horsfall-Bauer units.

The respiratory disease agents used in these experiments were IBV, Crawley virus, and PPLO. The IBV strain was isolated from a Virginia flock and serially passed in the allantoic sacs of 10-day

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TABLE 2—Correlation Between Salpingitis and Various Sites of Infection in 43 Female Chickens, 3 to 6 Weeks Old *

	Salpingitis	No salpingitis
LGA† infection	24	3
No LGA infection	0	16
LPT** infection	21	10
No LPT infection	3	9
RGA‡ infection	9	9
No RGA infection	15	10
Pericarditis	16	10
No pericarditis	8	9

* Inoculated in either the left or right posterior thoracic air sacs with a 24-hour tryptose broth culture of either the *b* or *c* type of *Esch. coli*; † LGA = left greater abdominal air sac; ** LPT = left posterior thoracic air sac; ‡ RGA = right greater abdominal air sac.

embryonating eggs. The strain produced slight respiratory changes in 3- to 6-week-old chickens after aerosol exposure. The allantoic fluid used contained about 10^5 e.l.d.$_{50}$/0.1-ml. dose. The Crawley virus strain also was isolated from a Virginia flock and propagated in the allantoic sacs of 10-day embryonating eggs. This strain produced mild signs after air sac inoculation of allantoic fluid containing about 10^4 e.l.d.$_{50}$/0.1-ml. dose. The strain of PPLO has been referred to previously as the "W" strain.[2, 3] This strain was isolated from a flock of Virginia chickens and serially passed in the yolk sacs of 7-day embryonating eggs. The infective yolk used in these experiments contained about $10^{4.8}$ e.l.d.$_{50}$/0.1-ml. dose.

EXPERIMENTS AND RESULTS

Production of Salpingitis.—To investigate the production of salpingitis, 43 female chickens, 6 weeks old, were inoculated in either the left or right posterior thoracic air sac with an 18-hour tryptose broth culture of *Esch. coli* type *c*. The birds were necropsied three weeks after inoculation. The results (table 2) indicate that, when the left greater abdominal air sac was also infected, salpingitis resulted in almost all females. No correlation was shown between salpingitis and pericarditis or right greater abdominal air sac infection. There was some correlation between salpingitis and left posterior thoracic air sac infection.

TABLE 3—Results of Inoculating 20 Chickens, 2 Months Old, in the Left Lesser Abdominal Air Sacs with a 24-Hour Tryptose Broth Culture of the c Type of Escherichia Coli

Time of death*	Distribution of lesions		
	Heart	Air sac	Oviduct
1 to 3 days	4/4	4/4	0/4
3 days to 1 month	5/5	5/5	4/5
1 to 2 months	0/1	0/1	1/1
3 to 4 months	1/4	2/4	3/4
4 to 5 months	0/2	0/2	2/2
Killed at end of fifth month	0/2	0/4	3/4

* After inoculation.

A group of 20 female chickens, 5 weeks old, was inoculated in the left greater abdominal air sac with 0.1 ml. of a 24-hour tryptose broth culture of *Esch. coli* type *c*. They were isolated for six months, and all birds which died were necropsied. The coliform organism isolated from the oviduct was serologically typed. While the oviducts of birds which died less than one month after inoculation were less than 8 mm. in diameter, the oviducts gradually increased in size until they were 6 cm. in diameter five months after inoculation. In contrast to infections of the heart, liver, and air sac, which are usually terminated within two weeks in nonfatal cases, oviduct infection was found to last over five months, probably resulting in permanent nonfunction.

These experiments indicate that salpingitis probably is produced by direct spread of the bacteria from the left greater abdominal air sac, through the attached short mesosalpinx, into the oviduct.

TABLE 4—The Effect of Inoculating Small Numbers of Escherichia Coli Bacteria, Alone or with the W Strain of PPLO, into the Air Sacs of 5-Week-Old Chickens

No. of bacteria inoculated	Types of *Esch. coli* without PPLO			Types of *Esch. coli* with PPLO		
	b	*c*	*d*	*b*	*c*	*d*
10^1	0*	1	0	1	3	5
10^2	0	3	3	2	4	5
10^3	1	4	2	3	4	4
10^4	1	3	5	3	4	5

* Number of birds (out of 5 inoculated) with pericarditis or serological titers over 2^5.

Air Sac Inoculation with Esch. Coli.—Experiments were performed to determine the number of viable *Esch. coli* bacteria necessary to produce infection after air sac inoculation. Groups of 5 chickens, 5 weeks old, were inoculated in the air sacs with 10^1, 10^2, 10^3, or 10^4 bacteria, in tryptose broth, of the *b*, *c*, or *d* types alone or with yolk-propagated PPLO. The 10^1 dose was 3, 7, or 11 bacteria, as determined by a pour-plate method, of the *b*, *c*, or *d* types.

A bird was considered to be infected if it had pericarditis on necropsy, or had an O serological titer of over 2^5 for the type inoculated. The results (table 4) indicate that types *c* and *d* required fewer viable bacteria than type *b* to produce infection, that PPLO greatly reduced the number of

TABLE 5—Mortality Following the Inoculation of Various Strains of PPLO, in Yolk or Mediums, and Various Types of Escherichia Coli, in Tryptose Broth, into the Posterior Thoracic Air Sacs of 6-Week-Old Chickens

Strains of PPLO	Strains of *Esch. coli*			
	b	*c*	*d*	Total*
W	1†	4	5	10
Medium-passed W	2	7	7	16
I	0	7	5	12
H	1	8	6	15
No PPLO	0	3	3	6
Total**	4	29	26	

* Out of 30 inoculated; † out of 10 inoculated; ** out of 50 inoculated.

bacteria required, and that as few as 10 viable bacteria can produce infection via the air sacs. In a previous experiment,[4] it was found that about 500,000 viable bacteria were necessary to produce infection when administered intravenously. Despite several attempts, pericarditis could not be produced in PPLO-infected chickens by placing pathogenic types of *Esch. coli* in the drinking water. However, pathogenic types were often maintained in the lower intestinal tract for over three months.

Only the W strain of PPLO was used in most of these experiments. It was not certain whether the increased activity of *Esch. coli* in the presence of this material was unique with this strain or whether an unknown agent in the egg-propagated material might be a factor. To investigate this, four strains of PPLO (H, I, and W strains passed in yolk and the W strain in the 55th medium passage) were inoculated into the air sacs of 6-week-old chickens with the *b*, *c*, or *d* types of *Esch. coli*. The medium used was Difco PPLO broth to which 0.25 per cent dextrose, 1.0 per cent yeast hydrolysate, and 1.0 per cent PPLO serum fraction were added.

The results (table 5) indicated that medium-propagated PPLO increased mortality as least as much as did the yolk-propagated strains, and that *Esch. coli* type *b* did not produce as much mortality as types *c* and *d*. The results also indicated that at least three strains of PPLO can increase *Esch. coli* pathogenicity. The increased mortality with the W strain in medium indicated that PPLO alone can increase *Esch. coli* activity.

Aerosol Exposure to Esch. Coli.—A series of experiments was conducted to determine the effect of exposing birds to aerosols of broth cultures of *Esch. coli*. Aerosols were produced by a No. 40 DeVilbiss nebulizer with 4 lb. of air-line pressure. Birds were exposed to the aerosols in 8-cu. ft. Horsfall unit cages without modifying the air flow. The nebulizer was run for one minute, during which approximately 2 ml. of material was discharged into the cage.

In one experiment, groups of 5-week-old turkey poults were inoculated in the air sacs with the yolk-propagated W strain of PPLO. At intervals, groups were exposed to aerosols of *Esch. coli* type *c*. The results (table 6) indicated that the greatest susceptibility occurred in the group exposed six days after PPLO infection. Simultaneous studies of the microscopic changes in the air sacs indicated that this also was the time of strongest lymphoid reaction.

TABLE 6—The Effect of Exposure of 5-Week-Old Turkeys to Aerosols of the c Type of Escherichia Coli at Various Times After Air Sac Inoculation with the W Strain of PPLO

	Day of aerosol exposure to *Esch. coli* after PPLO inoculation			
	0	2	6	16
No. developing pericarditis per No. exposed	1/15	2/15	7/13	1/12

Five-week-old chickens were exposed to Crawley virus by an aerosol and by injection into the air sacs. Groups of these birds were exposed to aerosols of *Esch. coli* type *d* at the same time, one half, one, two, four, eight, or 11 days later. In another experiment, chickens were similarly exposed to IBV.

The results (table 7) indicated that

TABLE 7—The Effect of Exposure of 5-Week-Old Chickens to Aerosols of the d Type of Escherichia Coli at Various Times After Exposure to Either Crawley Virus or IBV

	Day of aerosol exposure to *Esch. coli* after IBV or Crawley virus exposure								
	0	½	1	2	4	8	11	16	22
Crawley virus	0/6*	1/6	1/6	2/6	0/6	0/6	....	....	....
IBV	0/5	0/5	1/5	0/7	0/7	0/7	4/7	3/7	3/7

* Numerator = No. developing pericarditis; denominator = No. exposed.

TABLE 8—Pericarditis-Producing Ability of Various Numbers of the d Type of Esch. Coli When Administered in an Aerosol to 5-Week-Old Chickens Three, Four, Five, and Eight Days After Exposure to IBV, PPLO, and Crawley Virus

Viable cells in aerosol*	Exper. 1	Exper. 2	Total
10^2	4/7†	2/5	6/12
10^4	0/6	0/6	0/12
10^6	2/6	2/6	4/12
10^8	3/6	1/6	4/12

* Total viable bacteria by pour-plate method; † numerator = No. with pericarditis, and denominator = No. exposed.

Crawley virus-infected chickens are most susceptible to *Esch. coli* aerosols two to four days after inoculation, and that IBV-infected chickens are most susceptible 11 to 22 days after inoculation. Jungherr [7] has reported that, between 12 to 18 days after inoculation, chickens infected with IBV have follicular or diffuse lymphoid reactions in the propria of the trachea. A lymphoid reaction is prominent in the air sacs of Crawley virus-infected chickens about two to four days after inoculation. These observations indicate that an intense lymphoid reaction may have something to do with the production of pericarditis by *Esch. coli* aerosols.

In one experiment, three groups of 9 turkeys, 5 weeks old, were inoculated in the air sacs with 0.1 ml. of the yolk-propagated W strain of PPLO. Several days later, they were exposed to aerosols containing 10^5, 10^7, or 10^9 viable bacteria. At necropsy five days later, 4 birds in each group were found to have developed pericarditis.

Air Sac Inoculation with Mixed PPLO, Crawley Virus, IBV, and Esch. Coli.—In two other experiments, 5-week-old chickens were exposed to aerosols of an undiluted mixture of allantoic fluid and yolk containing Crawley virus and PPLO. Groups of these were exposed three, four, five, and eight days later to aerosols containing 10^2, 10^4, 10^6, or 10^8 viable cells of *Esch. coli* type *d*. Necropsy, five days after the last exposure, indicated (table 8) that there was no increase in the incidence of pericarditis as the dose was increased. The reason for the absence of pericarditis at the 10^4 dosage level was not evident. Similar results have been reported by Parry [8] for *Pasteurella pestis* infection in mice.

In another series of experiments, groups of 5-week-old birds were exposed to aerosols of undiluted 18-hour tryptose broth cultures of *Esch. coli* type *d*, one half, one, two, three, four, six, and eight days after exposure to various combinations of IBV, Crawley virus, and PPLO. In the first experiments, the allantoic fluid containing IBV or Crawley virus and yolk containing the W strain of PPLO were mixed and administered by means of a nebulizer. In the second experiment, the mixture of respiratory disease agents was given into the air sacs. In the third experiment, the respiratory disease agents and *Esch. coli* were mixed and injected into the air sacs as a single exposure.

TABLE 9—Effect of the d Type of Esch. Coli on Birds Exposed to IBV, Crawley Virus, and PPLO Alone or in Various Combinations

	Experiment				
	1*	2†	3**	Total	Percentage
PPLO	4/12‡	1/9	5/13	9/34	29
Crawley virus	5/12	2/9	4/12	11/33	33
IBV	1/12	4/8	0/5	5/25	11
PPLO + Crawley virus	2/12	3/9	6/11	11/32	34
PPLO + IBV	2/12	4/9	1/6	7/27	17
IBV + Crawley virus	3/12	3/8	0/6	6/26	14
All agents	5/12	6/9	2/6	13/27	48
Esch. coli alone	1/12	1/9	1/11	3/32	9
Total	23/96	24/70	19/70	65/236	28

* Respiratory agents, aerosol, *Esch. coli* aerosol one half, one, two, three, four, five, six, seven, eight, and nine days later; † respiratory agents, air sac, *Esch. coli* aerosol one half, one, two, three, four, six, and eight days later; ** respiratory agent and *Esch. coli* into air sac at same time; ‡ numerator = No. with pericarditis, and denominator = No. exposed.

The results (table 9) indicate that all combinations of respiratory disease agents increased the pericarditis-producing ability of *Esch. coli*.

SUMMARY

Certain types of *Escherichia coli* are important complicating factors in chronic respiratory disease (CRD) and other respiratory diseases.

The aerosol route appears to be the easiest route of infection and is probably most important naturally. At certain periods in the course of respiratory diseases, chickens or turkeys are more susceptible to *Esch. coli* aerosols. These periods are characterized by increased lymphoid proliferation in the respiratory tract lesions. At the periods of greatest susceptibility, small numbers of *Esch. coli* bacteria in an aerosol can produce pericarditis, and increasing the concentration of *Esch. coli* in the aerosol does not increase the incidence of pericarditis. The injection of as few as seven *Esch. coli* bacteria into the air sac can result in infection.

The incidence of pericarditis can be increased by adding India ink, pleuropneumonia-like organisms (PPLO) propagated in yolk or medium, infectious bronchitis virus (IBV), or Crawley virus to the inoculum, or by increasing the concentration of *Esch. coli* bacteria.

Salpingitis is a serious, long-lasting complication which follows *Esch. coli* infection of the left greater abdominal air sac.

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Symposium on Chronic Respiratory Diseases of Poultry. III. Epizootiology of Chronic Respiratory Diseases in Chickens

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CHRONIC RESPIRATORY DISEASE (CRD) is widespread among poultry in the United States and Canada and is of great economic importance to the poultry industry. In 1943, Delaplane and Stuart [5] first reported that the salient features of CRD were nasal discharge, respiratory râles, slow rate of spread, moderate decline in egg production, anorexia, and loss in body weight. They associated it with a viral-like agent that could be propagated in embryonating eggs and in the natural host. More recent investigations [1, 3, 4, 6, 8, 12-16, 18, 20-23] have added to our knowledge of CRD, with respect to cause, host susceptibility, symptomatology, pathology, transmission, immunity, and control.

NATURE AND TRANSMISSION

Some controversy still exists regarding the status of CRD as a definite disease entity, its specific cause, and its importance as a distinct respiratory infection.[7, 9] However, several investigators have attributed it to a specific pleuropneumonia-like organism (PPLO) that is capable, when inoculated into susceptible chickens, turkeys, and other birds, of reproducing the syndrome and pathological picture of uncomplicated CRD as observed in commercial flocks.[2, 10, 15, 17, 18]

It is recognized that in many respiratory disturbances, especially among broilers, there may be complicating multiple infections, both viral or bacterial.[11, 14, 20] Investigations have demonstrated that multiple infections similar to those observed in the field can be reproduced in the laboratory.[11] Naturally occurring CRD, when complicated with other infections, frequently results in significant mortality whereas, in uncomplicated CRD, the mortality is insignificant or slight. The authors have reproduced the disease in several thousand chickens and turkeys by artificial exposure without producing significant mortality.[21-23]

When infections are induced with the CRD agent collected from birds with natural cases, the pathological changes in the various tissues occur in the following decreasing order of frequency: trachea, air sacs, sinuses, and lungs.[20] In the natural disease, these tissue sites are also involved most frequently and show the greatest change.[22, 23] As the disease progresses, one may observe striking changes, particularly in the air sacs, varying from a slight translucency to a marked opacity and thickening of the membranes, which may be coated with whitish yellow exudate of differing degrees of turbidity and consistency. Severe aerocystitis is observed most frequently in young birds.

The disease may occur in chickens of all ages, the losses being greatest among birds 5 to 16 weeks old, manifested by mortality, poor growth, and rejection in poultry dressing plants.[14, 19]

In mature birds, the disease may cause a moderate decline in egg production, loss in body weight, and decrease in hatchability.

The manner in which CRD may be spread is of great concern. It was first believed that the transmission of the disease was limited in scope. However, it has been found that the agent may be spread by direct contact within a flock, or from flock to flock on the same premises by mechanical carriers.[23] The most common mode of transmission is from the dam to the chick via the egg. In order to control CRD, a greater knowledge of its spread seems urgent. In this paper, the authors report experimental data concerning this phase of the disease.

MATERIALS AND METHODS

Egg Transmission of CRD.—Studies were conducted in two commercial pedigreed breeding flocks in which CRD had been identified by laboratory examination.

Trial 1.—In the fall of 1953, with the cooperation of the owner, a small group of White Leg-

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Part of the investigation concerning egg-transmission studies was made possible through the cooperation of Dr. Edward F. Godfrey, geneticist, Mount Hope Poultry Farm, Williamstown, Mass. Appreciation is also extended to the American Cyanamid Co., New York, N.Y., for the chlorotetracycline (aureomycin), and to Merck and Co., Inc., Rahway, N.J., for the streptomycin sulfate and dihydrostreptomycin sulfate used in these studies.

horn (WL) pullets and cockerels were selected to supply hatching eggs for egg-transmission studies at the laboratory. The pullets were selected from 430 dams, on the basis of the presence of CRD lesions in embryos, and in culled chicks from a series of six hatches obtained from the 430 dams. Dams which had produced embryos and chicks with CRD lesions were placed in isolation on the farm, and hatching eggs from them were sent to the laboratory over a period of seven months for weekly or biweekly settings. The eggs were identified by dam number. At the end of each hatching period, all pipped eggs and chicks were examined for CRD lesions. As facilities permitted, chicks from certain hatches were hatched and raised in isolation. At 4 to 5 weeks of age, the chicks were vaccinated with either Newcastle disease (ND) or infectious bronchitis (IB) live virus vaccines. The chicks were examined carefully before and after this vaccinating for evidence of CRD infection. At 8 to 10 weeks of age, the chicks were killed and examined for lesions of CRD. Embryo and bird inoculations were made with materials collected from some affected birds. At the end of the study, 12 dams were examined for evidence of CRD infection.

Trial 2.—In the fall of 1954, a second flock on this same farm was selected, on the same basis, from a group of 480 dams. The procedure was similar to that of the previous year, except that it continued for 11 months and part of the chicks selected for raising were not stressed by vaccination but were maintained in separate isolation units. At the end of the hatching period, 10 dams were submitted to the laboratory for CRD tests.

Trial 3.—On this same farm, in the fall of 1955, two groups of 100 pullets with 8 males each, serologically positive for CRD, were isolated in two separate buildings with separate attendants and were investigated for eight months. Group A was placed on a regimen of antibiotic treatment. Chlorotetracycline-medicated feed (500-Gm. level) was fed during the first part of the hatching period. The birds also were given streptomycin sulfate (125 mg.) and dihydrostreptomycin sulfate (125 mg.) for four monthly intramuscular injections followed by six weekly injections. Group C was not treated. Periodic serological tests were made and, at the end of the trial, specimens from each group were submitted for CRD tests. The procedure was similar to that of 1954, except that chicks from several hatches were raised to sexual maturity on three nonpoultry farms and were tested periodically for CRD by the rapid serum-plate test. Hatching eggs were obtained from birds on two of these farms to produce second generation flocks and to examine for CRD lesions in embryos and day-old chicks.

Trial 4.—In the summer of 1956, another commercial pedigreed flock (White Plymouth Rock) of 2,100 birds was investigated for the purpose of establishing a CRD-free progeny flock. All of the birds were given one intramuscular injection of streptomycin sulfate (125 mg.) and dihydrostreptomycin sulfate (125 mg.); subsequently, 450 old hens and 100 yearling hens were treated weekly from May 7 to Aug. 9, 1956. Hatching eggs were saved, beginning one week after the first injection and continuing at approximately one-week intervals until one week after the last treatment. Fourteen hatches were obtained and brooded separately. Culled chicks and embryos from pipped eggs from a few hatches were submitted to the laboratory for examination. The first five hatches were brooded for five to six weeks and then moved to range. The remaining nine hatches were brooded and raised in confinement. All the chicks were vaccinated at 5 to 10 weeks of age with ND and IB vaccines administered in the water. Nine of the later hatches contracted a mild form of IB at 4 to 6 weeks of age. When 12 to 14 weeks old, the birds were vaccinated for fowlpox by the stick method. At 4 to 5 months of age, they were revaccinated with ND and IB vaccines administered in the water. At an early age, a random sample of each hatch was tested for CRD with the rapid serum-plate test, using PPLO antigen. All birds in the flock were tested repeatedly after they had attained sexual maturity.

Trial 5, Transmission of CRD by Cohabitation.—The natural spread of CRD was investigated in ten commercial flocks that were vaccinated with combination ND and IB dust vaccine * at 4 to 5 weeks of age and revaccinated at 16 to 20 weeks of age. Approximately five weeks after the last vaccination, susceptible, CRD-free, mature chickens were placed in contact with the vaccinated birds. Spread of CRD infection among the vaccinated and control birds was determined by periodic tests using the rapid serum-plate method and PPLO antigen.

RESULTS

Trial 1.—In 1953, 1,588 embryos from the 430 WL pullets were examined, of which 7.5 per cent had CRD lesions (table 1). The incidence of lesions was greater among the dead than among the living embryos. Lesions of CRD were found in 2.8 per cent of 640 culled chicks examined. Eighty-one dams were identified as producing embryos and chicks with CRD lesions in the six hatches examined. It is of interest that, while the majority of positive dams were detected in the first three hatches, evidence of CRD appeared in all six hatches.

Of the 81 positive dams, 66 were isolated in a building on the farm, together with 5 males, to form a breeding unit from which 23 groups of chicks were hatched from Dec. 31, 1953, to July 23, 1954. In 13 of the 23 hatches, all chicks at 1 day of age, as well as all nonpipped and pipped eggs, were examined for CRD lesions (table 2).

* Biolater, produced by the American Cyanamid Co., New York, N.Y.

TABLE 1—Incidence of CRD Lesions in Embryos and Culled Chicks Obtained from a Flock of 430 Pullets (1953)

Date of hatch	Embryos						Culled chicks		No. dams positive
	Dead		Alive		Total				
	(No.)	(% pos.)	(No.)	(% pos.)	(No.)	(% pos.)	(No.)	(% pos.)	
11/18/53	42	19.0	262	3.1	304	5.3	79	2.5	15
12/10/53	75	29.3	280	7.9	355	12.4	127	3.1	26
12/30/53	58	20.7	280	7.1	338	9.5	129	3.9	17
1/20/54	66	13.6	194	4.1	260	6.5	149	2.0	10
2/10/54	31	12.9	98	2.0	129	4.7	156	2.6	9
3/3/54	30	3.3	172	1.7	202	2.0	0	....	4
Total	302		1,286		1,588		640		81
Average		18.5		4.9		7.5		2.8	

The high rate of infertile eggs and dead germs was attributed largely to the condition of the flock, as determined by a controlled setting in another incubator. Interestingly, the incidence of CRD lesions was much less among the embryos from nonpipped eggs than among embryos of pipped eggs and among day-old chicks. This confirms previous reports of artificially and naturally infected embryos.[21,22] The incidence of CRD lesions in embryos from pipped eggs varied significantly among hatches, but revealed no definite trend for the hatching period. Among the day-old chicks, the incidence of lesions was relatively constant for the different hatches.

Data on 10 hatches, from the 66 positive dams, from which chicks were raised in isolation to 8 weeks of age, are listed (table 3). At approximately 4 weeks of age, all chicks were vaccinated either with ND virus by wing-web vaccination or by intratracheal inoculation with IB virus. The incidence of CRD lesions among the embryos in the pipped eggs was similar to that among embryos, as listed (table 2). Definite signs of CRD, primarily nasal discharge and tracheal râles, were observed in seven of the ten hatches of chicks. The lesions were largely confined to the upper respiratory tract, although typical aerocystitis was observed in several chicks. In two hatches, the CRD infection was complicated with *Escherichia coli* infection expressed, primarily, as a serofibrinous pericarditis. A fibrinous perihepatitis was observed in a few birds; pure cultures of *Esch. coli* were isolated from these birds. In hatches 2, 10, 13, and 19, the percentage of positive chicks ranged from 50.0 to 75.1.

The CRD agent was isolated by bird inoculation from chicks in six of the ten hatches.

Typical signs and lesions of CRD were produced in chickens and turkeys inoculated with material harvested from the positive chicks in hatches 2, 7, 10, 13, 16, and 19. The CRD agent was isolated in turkeys and chickens from a 4-day-old chick in hatch 4.

As the hatching progressed, the inci-

TABLE 2—Incidence of CRD Lesions in Embryos and Chicks Obtained from the 66 Positive Dams (1954)

Hatch No.	No. eggs set	Infertile eggs and dead germs (%)	Embryos from eggs				Day-old chicks	
			Nonpipped		Pipped			
			(No.)	(% pos.)	(No.)	(% pos.)	(No.)	(% pos.)
5	197	34.5	33	0	28	33.3	68	11.8
6	289	28.0	41	7.3	63	7.9	104	2.9
8	206	37.8	16	0	49	22.4	63	3.2
9	300	21.6	45	4.4	55	12.7	135	1.5
11	246	43.9	40	5.0	29	27.6	69	4.3
12	199	31.7	38	0	26	7.7	72	8.3
14	228	39.5	33	0	29	6.9	76	6.6
15	269	37.1	32	0	42	19.0	95	7.4
17	221	24.9	24	0	16	18.8	126	6.3
18	168	37.5	25	0	16	18.8	64	3.1
20	209	50.2	23	0	22	0	59	5.1
21	162	39.5	7	0	9	11.1	82	4.9
23	135	29.6	6	0	12	33.3	77	7.8
Total	2,829		363		396		1,090	
Average		34.6		1.1		14.9		5.4

dence of CRD lesions did not vary significantly between embryos and chicks. The hatching data and incidence of CRD lesions for each of the 12 dams that produced an appreciable number of positive embryos and chicks are given (table 4). Some dams produced positive embryos throughout the period, others did not. Since these dams were originally selected on the basis of producing CRD lesions in embryos and chicks, it is evident that such lesions may occur irregularly over a period of time but seldom at a high rate.

The results of the 23 hatches from the 66 positive dams are summarized briefly (table 5). The hatching trials demonstrated definitely that the CRD agent is egg-transmitted and is capable of initiating the various forms of the disease as seen in the field.

At the end of the hatching period, 12 of the 66 dams were submitted to the laboratory. Mild air sac lesions were found in 8. Typical signs and lesions of CRD were produced in turkeys that were inoculated with broth suspensions of nasal swabbings obtained from the 12 birds.

Trial 2.—In the fall of 1954, a breeding unit of 76 birds, positive for CRD, was selected from 480 WL pullets. The flock was maintained in a manner similar to that of the previous year. A total of 21 hatches was obtained, the first on Dec. 30, 1954, and the last on Nov. 1, 1955.

The number of eggs set, hatching data, and the number of embryos and day-old chicks examined in each hatch are shown (table 6). The increased infertility and dead germs observed as the hatching period progressed were attributed to the diminished potency of the males and the condition of the flock rather than to CRD. The incidence of CRD lesions in the embryos and day-old chicks was slightly greater than observed the previous year. No seasonal variation was seen in the incidence of lesions. Of a total of 7,933 eggs set, 18.4 per cent were infertile or revealed dead germs. The incidence of CRD lesions was 16.9 per cent for 917 embryos from pipped eggs, 3.9 per cent for 557 embryos from nonpipped eggs, and 7.6 per cent for 4,277 day-old chicks.

From the series of 21 hatches of chicks, some chicks from six hatches were raised in isolation units up to 8 to 10 weeks of age. At 4 weeks of age, the chicks from each hatch were allotted to two equal groups, one being stressed with IB vaccination and the other maintained as a control.

The CRD findings in each hatch are summarized (table 7). A total of 328 chicks was raised in the control groups, in which no signs were observed; but, on necropsy at the end of the observation period, 7 chicks of two hatches manifested lesions suggestive of CRD. However, in one case, material from the suspicious chicks was inoculated into CRD-free birds with negative results. The birds in the last four hatches were tested with the serum-plate method, using PPLO antigen, with negative results.

In the stressed group, 364 chicks were raised, of which 16 manifested signs in two hatches, and 86 revealed lesions in five hatches. Marked lesions were detected only

TABLE 3—Egg Transmission of the CRD Agent from Naturally Infected Dams to Their Progeny (1954)

Hatch No.	No. eggs set	Infertile eggs and dead germs (%)	Incidence of CRD lesions among specimens examined								
			Embryos from eggs				Day-old chicks		8-week-old chicks		CRD isolation*
			Nonpipped		Pipped						
			(No.)	(% pos.)	(No.)	(% pos.)	(No.)	(% pos.)	(No.)	(% pos.)	
1	107	22.4	18	0	31	9.7	3	0	31	0	—
2	262	29.0	109	3.7	28	42.9	0	0	48	50.0	+
3	367	42.7	44	4.5	65	12.3	0	0	101	4.0	—
4	452	43.8	90	2.2	51	2.0	14	0	99	11.0	±†
7	277	33.2	50	8.0	59	5.1	14	0	62	46.8	+
10	285	23.5	33	9.1	51	19.6	4	25.0	130	58.5	+
13	236	36.9	34	0	28	21.4	10	10.0	77	75.1	+
16	192	42.2	19	0	23	21.7	6	16.7	63	25.4	+
19	204	28.0	3	0	17	47.1	4	0	123	74.8	+
22	195	47.7	27	0	21	4.8	6	33.0	48	4.2	—
Total	2,577		427		374		61		782		
Average		36.2		3.5		15.2		8.2		39.8	

* CRD agent isolation based on bird inoculation; † CRD agent isolated in turkeys and chickens from a 4-day-old chick.

TABLE 4—Summary of Hatching Data and CRD Findings in Selected Eggs from 12 Naturally Infected Pullets (1954)

Dam No.	No. eggs set	Fertility (%)	No. chicks hatched	Hatchability (%)	No. day-old chicks examined	Incidence of CRD lesions in day-old chicks and embryos by settings																	
						1–5			6–10			11–15			16–20			21–23			Totals		
						C	P	S	C	P	S	C	P	S	C	P	S	C	P	S	C	P	S
21	152	97.4	95	64.2	57	0	0	0	0	1	1	2	0	0	0	1	0	0	1	0	2	3	1
27	89	96.6	37	43.0	26	0	0	0	0	0	0	3	0	0	2	1	0	1	0	0	6	1	0
48	79	82.3	14	21.5	11	1	4	0	1	6	3	0	5	3	0	0	0	0	0	0	2	15	5
208	125	93.6	75	64.1	44	0	1	0	0	0	0	1	1	0	1	0	0	2	0	0	4	2	0
223	101	82.2	39	47.0	18	0	2	0	0	2	0	0	1	0	0	0	0	0	0	0	0	5	0
248	66	84.8	23	41.1	14	0	1	0	0	0	0	0	3	0	1	0	0	1	0	0	2	4	0
264	50	86.0	16	37.2	5	0	2	0	0	1	0	0	3	0	0	2	0	1	0	0	1	8	0
280	113	82.3	28	30.1	22	0	1	0	0	3	0	0	1	0	0	1	0	0	0	0	0	6	0
289	79	92.4	21	28.8	14	1	2	0	0	9	0	2	1	0	4	4	0	0	2	0	7	18	0
328	112	92.9	43	41.3	25	0	0	0	0	5	0	0	1	0	0	0	0	0	0	0	0	6	0
365	102	78.4	15	18.8	8	0	2	1	1	0	0	0	1	0	0	0	0	0	0	0	1	3	1
399	135	89.6	41	33.9	19	1	3	0	1	0	0	0	0	0	0	0	0	0	0	0	2	3	0
Total	1,203		447		263	3	18	1	3	27	4	8	17	2	8	9	0	5	3	0	27	74	7
Average		88.9		41.8																			

C = day-old chicks; P = embryos from pipped eggs; S = embryos from nonpipped eggs.

among the birds in hatch 10. Materials collected from birds with lesions and verified by bird inoculation yielded positive results for hatches 5, 10, and 13; the findings in hatch 8 were questionable. Serologically positive birds were detected only in hatch 10.

At the end of the hatching period 10 dams were examined at the laboratory; all gave evidence of CRD by culture, bird inoculation, and serological tests.

Trial 3.—The results of the 1955 series of hatches were similar to those of the 1954 series, although the degree of transmission appeared to be less (table 5). It is significant that CRD-free progeny can be raised from infected dams. Also, it is apparent that a stressing mechanism may help to precipitate the disease, thus confirming observations made in the field.

The series of 19 hatches obtained from flocks A and C extended from Jan. 18 to July 8, 1956. The hatching data and incidence of CRD lesions for groups A and C are summarized (table 8). The incidence of CRD lesions in the embryos and day-old chicks is less in the nontreated group than in the treated flock. However, neither group of embryos or chicks had a high incidence of CRD lesions. Seasonal variation in the incidence of CRD lesions was not detected.

Chicks were raised from both groups in hatches 1, 3, 5, 7, 9, 10, 11, 16, 18, and 19. They were maintained in animal isolation quarters with a separate room for each group. Approximately half the chicks in hatches 1 and 3 were stressed by debeaking and with diethylstilbestrol; one half were maintained as controls. The stressed groups were raised in rooms separate from the controls. In the remaining eight hatches, each consisting of chicks from the two breeding units, approximately half were vaccinated with IB, and the others were maintained as controls in separate units. Unfortunately, failures occurred in maintaining some of the control groups free of IB. Hatches 1 to 13 were obtained from group A during the period that these birds were given four monthly injections of streptomycin (Feb. 14, March 15, April 13, and May 3). Hatches 14 to 19, inclusive, were obtained while flock A was being treated weekly with streptomycin (June 4 to July 9).

Data on all hatches and on chicks raised in isolated quarters at the laboratory are

TABLE 5—Egg Transmission of CRD from Naturally Infected Dams

Items	1954	1955
No. hatches	23	21
Total eggs set	5,406	7,933
No. embryos (nonpipped eggs)	790	557
Positive (%)	2.8	3.9
No. embryos (pipped eggs)	770	917
Positive (%)	15.1	16.9
No. day-old chicks examined	1,151	4,277
Positive (%)	5.6	7.6
No. chicks raised	782	719
No. hatches raised	10	6
No. hatches stressed*	10	6
No. positive (lesions)	9	5
No. positive (isolations) †	7**	4**
No. chicks stressed	731	364
Positive (%) (lesions)	40.4	23.6
No. hatches not stressed	0	6
No. positive (lesions)	....	2
No. positive (isolations)	....	0
No. chicks not stressed	....	328
Positive (%) (lesions)	....	2.2‡

* The term "stressed" designates ND or IB vaccination; † isolation studies made in chickens and turkeys; ** 1 questionable; ‡ 7 birds showed slight lesions.

summarized (table 9). Only one group of 62 stressed chicks in hatch 18, from the nontreated dams, revealed evidence of CRD. Lesions were found in 9 birds, and 14 were serologically positive for CRD. Bacteriological and bird inoculation tests confirmed the pathological and serological findings.

It is of interest that chicks in hatch 18 manifested CRD, since the embryos and day-old chicks did not show a high rate of lesions. Likewise, it is difficult to evaluate the benefit derived from the streptomycin therapy in reducing or preventing the shedding of the CRD agent in the egg.

At the end of the hatching period, the dams in both groups were tested serologically and found positive, and 10 from groups A and C were brought to the laboratory. On necropsy, only 1 bird (group A) had lesions. Chickens inoculated with pooled material collected from the nasal passages and air sacs yielded negative results. However, PPLO cultures were isolated from the nasal passages of several birds in both groups.

Concerning the CRD status of these chicks, part or all of the pullets and a few cockerels were raised to maturity on three nonpoultry farms for further study. A total of 121 pullets from hatch 5 was placed on farm 1 at 11 weeks of age; 490 pullets from hatches 7, 9, 10, and 11, and 22 males from hatch 11 were placed on farm 2 at 7 to 13 weeks of age; and 296 pullets from hatches 16, 18, and 19, and 12 males from hatch 16 were placed on farm 3 at 10 to 13 weeks of age. These birds were tested periodically during the following 7 to 12 months with the rapid serum-plate test, using PPLO antigen, and no definite reactors were detected. There was no evidence of CRD in chicks from several hatches from flocks 2 and 3, even when they were raised to 9 weeks of age and stressed with IB vaccination.

The results of this series of hatches indicate that CRD-free progeny can be raised from infected breeding stock whether or not the dams are treated with antibiotics.

TABLE 6—Incidence of CRD Lesions in Embryos and Chicks Obtained from 76 Naturally Infected Dams (1955)

Hatch No.	No. eggs set	Infertile eggs and dead germs (%)	Embryos from eggs				Day-old chicks	
			Nonpipped		Pipped			
			(No.)	(% pos.)	(No.)	(% pos.)	(No.)	(% pos.)
1	144	9.7	11	0	17	5.9	102	5.9
2	308	8.1	15	0	37	18.9	117	5.1
3	308	9.7	18	0	27	14.8	233	6.0
4	358	7.3	21	4.8	48	10.4	263	4.6
5	413	9.9	17	5.9	43	20.9	188	5.9
6	430	12.3	26	7.7	41	22.0	310	6.1
7	420	14.3	22	0	47	19.1	291	10.3
8	414	10.6	19	0	38	10.5	181	3.9
9	442	12.0	25	0	42	28.6	322	14.6
10	521	16.3	29	6.9	51	27.5	233	12.9
11	409	17.4	28	3.6	43	27.9	267	9.0
12	503	11.5	33	6.1	74	23.0	230	6.5
13	459	14.8	34	2.9	42	23.8	197	3.0
14	484	15.3	30	3.3	42	19.0	338	4.4
15	436	19.3	34	5.9	41	14.6	277	7.2
16	492	33.3	35	2.9	40	22.5	253	10.3
17	378	38.4	15	0	37	8.1	181	5.5
18	434	33.9	107	7.5	177	8.5	3	0
19	208	46.6	7	0	6	0	98	11.2
20	155	33.5	6	0	9	22.2	88	4.5
21	217	33.2	25	0	15	6.7	105	10.5
Total	7,933		557		917		4,277	
Average		18.4		3.9		16.9		7.6

TABLE 7—Incidence of CRD in 8-Week-Old Chicks Raised from Positive Dams (1955)

Hatch No.	Control birds					Stressed birds				
	No. chicks	No. positive			CRD† isolation	No. chicks	No. positive			CRD† isolation
		Signs	Lesions	Serology*			Signs	Lesions	Serology*	
2	56	0	5	NT	NT	54	0	16	NT	N
5	58	0	0	NT	NT	58	1	15	NT	P
8	61	0	2	0	N	68	0	2	0	Q
10	52	0	0	0	N	65	15	52	52	P
12	51	0	0	0	N	55	0	0	0	N
13	50	0	0	0	N	64	0	1	0	P
Totals	328	0	7	0		364	16	86	52	

* Rapid serum-plate test; † isolation studies made in chickens and turkeys.
NT = no test; N = negative; P = positive; and Q = questionable.

The CRD-free flocks produced a second generation that remained free of CRD to beyond 9 weeks of age. They manifested no post-vaccination complications at 4 weeks or older, as is frequently observed in birds in commercial flocks.

Trial 4.—The results in commercial flock 2 were most interesting. As the chickens were raised to maturity, random sample tests were conducted with the rapid serum-plate test, using PPLO antigen. These tests were negative for all hatches, with the exception of the hatch of July 27, 1956. When reactors were detected, the entire group was tested, and approximately 100 per cent reacted. Ten reactors were submitted to the laboratory for further study and the remainder of the hatch was removed from the farm.

When the replacement stock had been placed in its permanent laying quarters, the birds were tested for pullorum disease and CRD. The flock was distributed in 12 pens, with some pens having stock from four different hatches. The results of the CRD serological tests are recorded (table 10).

Birds in pens 7 to 10 were tested on Nov. 19, 1956, and 14 doubtful reactors were detected; they were negative on later tests and on culturing at the laboratory. The birds in these 4 pens were retested in February and April, with no conclusive evidence of CRD infection. Birds in pens 3 and 4 were first tested on Nov. 20, 1956, and again in February and April; the only suspicious reactor was negative on laboratory examination. All birds thus far tested were from the same hatches (May 29, 1956,

TABLE 8—Incidence of CRD Lesions in Embryos and Chicks Obtained from Naturally Infected Dams (1956)

Group*	No. eggs set	Infertile eggs and dead germs (%)	Embryos from eggs				Day-old chicks	
			Nonpipped		Pipped			
			(No.)	(% pos.)	(No.)	(% pos.)	(No.)	(% pos.)
A	5,374	18.8	296	2.4	403	6.2	2,481	5.1
C	5,367	16.9	159	0.6	181	7.2	2,856	1.8

* Group A treated with antibiotics; group C not treated.

TABLE 9—Egg-Transmission Studies in Serologically CRD-Positive Chickens (1956)

Items	Group A (treated)		Group C (nontreated)	
Hatches	1–13	14–19	1–13	14–19
No. eggs set	3,788	1,586	3,784	1,583
No. embryos examined	530	169	264	76
Positive (%)	5.7	1.2	4.5	2.6
No. chicks examined	1,771	710	1,957	899
Positive (%)	5.9	3.1	2.2	0.7
No. hatches raised	7	3	7	3
No. chicks raised	840	342	889	377
No. controls	412	166	433	186
Positive	0	0	0	0
No. stressed	409	167	439	190
Positive	0	0	0	*

* Nontreated stressed birds of hatch 18 revealed lesions typical of CRD. These birds were positive serologically and on follow-up studies in cultures and birds.

and June 1, 1956). The birds in pens 5 and 6 were first tested on Dec. 10, 1956, and 24 suspicious reactors were detected. Of these, 1 died and CRD infection was not found in the other 23. Birds of both pens were retested on Feb. 14, April 2, and April 24, 1957, and 7 reactors were detected among the males in pen 5 on the April 2 test. These males had been introduced into this pen as serologically negative birds which originated from a reserve supply of males that had harbored reactors on previous tests. All the males in this pen were sold and replaced with males from a negative source. Two weak reactors were found in pen 6 on the April 2 test, and were removed from the flock. The birds in pens 5 and 6 represented hatches of June 22, June 29, and July 6, 1956.

The birds in pen 1, hatched July 13 and 20, 1956, were first tested on Feb. 11, 1957. Of the 13 weak reactors, the 9 females were examined at the laboratory and 3 proved positive for CRD. The 4 male reactors were sold. The birds in pen 1 were retested on Feb. 26, March 11, April 3, and April 24, 1957, and 12 doubtful reactors were detected. Three of 7 birds reacting on the first two tests were submitted to the laboratory; 2 were positive and 1 was negative for CRD. The remaining doubtful reactors were removed from the flock and sold. The final test on the birds in pen 1 was entirely negative.

Birds in pen 2, representing four different hatches (Aug. 3, 10, 17, and 24, 1956) were tested on Feb. 11, 1957; a high proportion of pullets were reactors and all the pullets were removed from the premises. The percentage of infection among the males was low in comparison with the pullets. The nonreacting males were retained and, when retested on February 26, more than 50 per cent reacted. The entire pen of males was discarded.

Of the birds in pen 11, hatched Aug. 31, 1956, 17 reacted on the first complete test on Feb. 12, 1957. Nine of the 14 reactors examined at the laboratory were classified as positive for CRD. On the second test (Feb. 26), 14 reactors were detected and 9 of the 11 submitted to the laboratory were found positive for CRD. On the third test (March 11), 2 reactors were detected; they were positive on retest. When the fourth test (April 3) revealed a high percentage of reactors, indicating that the infection was spreading extensively, the entire lot was removed from the premises.

All birds in pen 12 were tested initially on Feb. 12, 1957. Of the 15 reactors found, 14 were submitted to the laboratory, where tests indicated that 9 were positive for CRD. On the second test (Feb. 26), 5 re-

TABLE 10—Results of PPLO Serological Tests in Progeny Obtained from an Antibiotic-Treated, CRD-Infected Flock

Pen No.	Hatching date	Sex	Results of serum-plate tests											
			First test			Second test			Third test			Fourth test		
			Date	No. tested	Reactors	Date	No. tested	Reactors	Date	No. tested	Reactors	Date	No. tested	Reactors
1	7/13, 7/20	F	2/11	304	9	2/26	287	5	3/11	273	2	4/3	259	0
		M	2/11	29	4	2/26	24	0	3/11	24	0	4/3	24	5
2	8/3, 8/10,	F	2/11	361*	192	.....	.....	..	.....	.....	..	.....	.....	..
	8/17, 8/24	M	2/11	28	3	2/26	23*	13	.....	.....	..	.....	.....	..
3	5/29, 6/1	F	11/20	317	0	2/18	299	0	4/2	265	0	.....	.....	..
		M	11/20	24	0	2/18	22	0	4/2	22	0	.....	.....	..
4	5/29, 6/1	F	11/20	237	1	2/18	217	0	4/3	212	0	.....	.....	..
		M	11/20	18	0	2/18	17	0	4/3	16	0	.....	.....	..
5	6/22, 6/29	F	12/10	372	16	2/14	320	0	4/2	302	0	4/24	296	0
		M	12/10	30	1	.....	.....	..	4/2	21*	7	4/24	21†	0
6	6/29, 7/6	F	12/10	370	6	2/14	370	0	4/2	363	1	4/24	362	0
		M	12/10	30	1	2/14	29	0	4/2	29	1	4/24	28	0
7	5/29, 6/1	F	11/19	156	4	2/15	147	0	4/4	142	0	.....	.....	..
		M	11/19	12	0	2/15	10	0	4/4	10	0	.....	.....	..
8	5/29, 6/1	F	11/19	158	3	2/15	145	1	4/4	135	0	.....	.....	..
		M	11/19	12	0	2/15	12	0	4/4	11	0	.....	.....	..
9	5/29, 6/1	F	11/19	158	1	2/15	144	0	4/4	135	0	.....	.....	..
		M	11/19	12	0	2/15	12	0	4/4	12	0	.....	.....	..
10	5/29, 6/1	F	11/19	158	6	2/15	135	0	4/4	127	0	.....	.....	..
		M	11/19	12	0	2/15	12	0	4/4	12	0	.....	.....	..
11	8/31	F	2/12	103	12	2/26	81	10	3/11	80	2	4/3	78*	24
		M	2/12	10	5	2/26	5	4	.....	.....	..	.....	.....	..
12	9/7	F	2/12	104	7	2/26	96	3	3/11	91	0	4/3	89*	24
		M	2/12	11	8	2/26	3	2	.....	.....	..	.....	.....	..

* Birds sold; † replacement males.

The birds (257 F and 16 M) in pen 1 were tested and found to be negative on April 24.

actors were found, and 4 were positive for CRD at the laboratory. No reactors were found on the third test, but the fourth test detected 24 reactors among 89 birds. All the birds were removed from the premises.

It is evident that the birds hatched from May 29 to July 6, 1956, and raised on the range did not manifest conclusive evidence of CRD infection, whereas the remaining hatches, starting with July 13, 1956, proved definitely positive for the disease. These results demonstrated that antibiotic therapy failed to control the transmission of the agent from the dam to the progeny. The presence of CRD lesions in embryos and culled chicks also substantiated the positive results found in the progeny. However, one may advance the explanation that the CRD agent concerned had become streptomycin-resistant with prolonged treatment. This aspect of the problem was not investigated.

It may be significant that none of the chicks manifested any signs of CRD during the growing period and that no visible signs of disease were detected in the positive birds or in the flock as the infection spread. However, it is clearly revealed that CRD may not be eradicated from a flock by serological testing and the removal of reactors. Once the infection is established, it appears impossible to eliminate it by testing. When infection appears to be absent, the agglutination test may be useful in determining the freedom of a flock from CRD. However, the true status of a flock can not be evaluated fully until more time has elapsed, even though all birds in the flock may have passed at least one negative test. Progeny raised from the serologically negative breeders have not revealed conclusive evidence of CRD. Some lesions suggestive of CRD were detected in embryos and culled chicks obtained from the serologically negative dams. The progeny raised from this flock have not revealed evidence of CRD infection to date.

Trial 5.—In the ten commercial flocks that were dust-vaccinated for IB and ND at approximately 4 to 6 weeks of age and revaccinated prior to sexual maturity, a random sample of birds was serologically tested prior to vaccination and periodically tested postvaccination. On prevaccination tests, the results were either completely negative or showed a few doubtful reactors. However, in the first postvaccination tests in eight of the ten flocks, birds were either definitely positive or suspicious for CRD infection. In some flocks, all tested birds reacted. Subsequent tests of these flocks continued to be positive, as is illustrated by flocks 1, 2, and 3 (table 11). Test

TABLE 11—Natural Transmission of CRD in Commercial Poultry Flocks

Flock No. & groups	Hatching date	Results of CRD tests listed by sampling dates								
1										
Vaccinated		June 8*	July 12	Sept. 10†	Nov. 19	Dec. 26	Feb. 6	Mar. 19		
birds	5/1/56	3/22‡	5/22	22/22	22/22	22/22	19/19	16/16		
Controls 10/18/56**	4/5/56	.....	.....	.....	4/22	15/22	19/19	17/17		
2										
Vaccinated		Mar. 12*	Apr. 16	June 20*	July 23	Sept. 18	Dec. 14	Jan. 16	Mar. 6	
birds	2/15/56	0/22	22/22	22/22	22/22	22/22	22/22	22/22	22/22	
Controls 8/1/56**	3/5/56	.....	.....	.....	.....	3/23	17/22	18/20	22/22	
3										
Vaccinated		April 3*	May 3	June 22†	July 19	Sept. 17	Dec. 5	Jan. 15	Mar. 13	
birds	2/22/56	1/22	22/22	22/22	22/22	21/21	20/20	19/19	17/17	
Controls 7/26/56**	3/5/56	.....	.....	.....	.....	9/21	4/20	9/19	9/19	
4										
Vaccinated		July 11*	Aug. 8	Oct. 15†	Nov. 14	Jan. 3	Feb. 14	Mar. 27	May 7	June 20
birds	6/6, 9/56	5/22	4/22	4/22	6/22	1/22	0/21	0/21	0/19	2/19
Controls 11/20/56**	4/5/56	.....	.....	.....	.....	0/22	0/21	0/21	0/19	1/19
5										
Vaccinated		May 17*	June 11	Aug. 15†	Sept. 14	Oct. 23	Nov. 27	Jan. 9	Mar. 5	
birds	4/13/56	0/22	0/22	10/22	0/22	0/23	0/23	0/21	0/14	
Controls 9/24/56**	4/5/56	.....	.....	.....	.....	0/22	1/23	0/21	0/15	

* Date of initial vaccination; † date of second vaccination; ** date controls introduced into flock; ‡ denominator = No. tested, numerator = No. reactors.

TABLE 12—Test Results of the Individual Control Birds for CRD

Bird No.*	Results of CRD tests listed by sampling dates				
	10/2/56	11/19	12/26	2/6	3/19
MQ 68187	0	0	2	..	..
68222	0	2	3	4	4
68255	0	0	2	4	1
68262	0	0	..	..	..
68268	0	0	±	2	3
68282	0	0	0	4	..
68286	0	..	0	4	3
68291	0	0	0	4	3
68303	0	..	±	4	2
68305	0	0	±	1	3
68307	..	0	..	..	..
68310	0	0	2	4	4
68367	0	0	±	4	..
68374	0	..	±	4	3
68381	0	0	±	..	..
68405	0	0	0	4	..
68412	0	±	3	4	4
68417	0	0	2	4	3
68419	0	0	..	..	..
68422	0	0	3	..	3
68424	0	0	0	4	3
68427	0	1	2	4	3
68428	0	0	±	4	..
68431	0	3	..	4	3
68434	0	..	0	4	3
68452	0	0	0	..	3

* Birds introduced into vaccinated flock 1 on Oct. 18, 1956.

Degrees of agglutination: 0 = none; ± = trace; 1 = slight; 2 = partial; 3 = incomplete; and 4 = complete.

results of only five flocks are given in this table, since the findings in the other five flocks were similar. In flock 4, some of the vaccinated birds reacted to the initial tests, but there were essentially no reactors on later tests. Flock 5 did not reveal any reactors in eight tests except in the third test, when weak reactors were observed. All the birds in this flock were tested in April, 1957, and no definite reactors were detected. This was one of a few flocks in which no conclusive evidence of CRD infection was found.

In the vaccinated flocks, susceptible CRD-free, mature birds were added approximately five weeks after the second vaccination. In all of the ten flocks, except flocks 4 and 5, the control birds became positive after several weeks' contact with the vaccinated birds (table 12). None of the control birds in flocks 4 and 5, and only part of those in flock 3 became reactors. Clinical signs of the disease were detected in some of the control birds after they had been in contact with the vaccinated, CRD-infected chickens.

It appears from these results that, if CRD infection exists in a young flock and the birds are vaccinated for ND and IB, the infection may become generalized in the flock, as demonstrated by serological testing. Although the rate of spread varied in different flocks, it appeared to occur rapidly in some cases. This also was true for the susceptible control birds introduced into CRD-infected flocks.

DISCUSSION

Some aspects of the epizootiology of CRD are well understood, whereas others need further study. Poultry pathologists, for the most part, have accepted the viewpoint that CRD is a specific disease entity caused by a PPLO. Some investigators maintain that CRD is a respiratory disturbance resulting from a complexity of causes. Our studies, supported by those of other investigators, reveal that the uncomplicated disease follows a definite clinical course, produces a typical pathological picture, and induces an antibody response which is detectable by serological means. All of the features can be regularly reproduced in the laboratory by inoculating susceptible chickens with the CRD agent.

One of the important aspects of CRD is its mode of transmission. The experimental data, collected over a period of three years, reveal that the agent is egg-transmitted and that this possibly is the most common means of perpetuating the disease. It is well established that PPLO may produce gross lesions specific for the infection in the terminal stages of incubation of embryos. The incidence of these embryo lesions may vary in different hatches from the same dams, as well as among embryos from a single dam. This also is true for day-old chicks.

Groups of chicks hatched from eggs laid by CRD-infected dams and raised in isolation experienced definite outbreaks of CRD that were similar to those in the field. While the mortality was insignificant, the clinical features and the pathological changes were similar to those seen in the field in uncomplicated CRD. It was also demonstrated that egg transmission of the disease did not occur consistently in every hatch of chicks. There is, apparently, no plausible explanation of why hatching trials yielded fewer positive transmission results as experiments were repeated in the three successive years.

Antibiotic therapy administered to CRD-infected dams did not appear to restrain the passage of the agent from the dam to

the egg, as confirmed by the presence of lesions in embryos and day-old chicks, and the occurrence of the disease in the older progeny. Under certain conditions, CRD-free progeny from infected dams can be maintained free of the disease for at least 12 months.

Sample testing of progeny with the serum-plate agglutination test, using PPLO antigen, may or may not detect infection in a flock. Failure to identify the infected birds at an early age permits the disease to spread through the flock. Once the disease is well established in a flock, it appears unlikely that the infection can be eliminated from the premises except by depopulation.

In some commercial flocks tested at 4 to 6 weeks of age with the serum-plate test, evidence of CRD was not detected by small sample testing. However, after stressing with vaccination for IB and ND, the disease appeared to spread rapidly, so that essentially all birds in the flock reacted to the test. When susceptible CRD-free control birds were added to such flocks, the disease frequently spread rapidly to the introduced birds. This further substantiates previous findings that in many flocks the disease spreads to most birds in the flock, and that this usually occurs before the birds reach sexual maturity. The rapid spread of the CRD agent in a flock makes the disease more difficult to eradicate.

SUMMARY

Various aspects of the epizootiology of CRD have been discussed. Among the modes of transmission, transovarian infection was a common mode of perpetuating the disease from one generation to the next. Antibiotics administered to infected dams did not appear to inhibit the elimination of the agent via the egg. Partial flock testing may be unreliable in determining the CRD-free status of the flock, consequently the infection may spread from the untested birds. Under the conditions which exist in commercial flocks, the disease may become widespread prior to sexual maturity of the birds. This creates a difficult control problem.

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Symposium on Chronic Respiratory Diseases of Poultry
IV. The Control of Chronic Respiratory Disease

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THIS DISCUSSION of the control of chronic respiratory disease (CRD) is concerned with a brief review of the pertinent literature and with certain experimental and field observations cited in support of the opinions set forth.

The recognition of pleuropneumonia-like organisms (PPLO) as a constant and recognizable etiological factor, together with its transovarian passage, by Van Roekel and Olesiuk [12] furnished the basis for this author [6] to propose an eradication program patterned along the lines of the program for pullorum disease. This central idea of control by eradication has maintained itself. However, since it proved both technically and economically impossible to segregate breeder flocks on the basis of serological tests for PPLO, emphasis has been placed upon recognizing clean offspring from infected breeders and maintaining this clean status.

The plan proposed by Crawley and Fahey [3] for the control of CRD by periodic injection of the breeding stock with streptomycin has been a major advance. Similar experiments by Adler *et al.*[1] indicated that egg-transmission of PPLO could be prevented by parenteral treatment of infected breeders with a mixture of streptomycin and dihydrostreptomycin. The relative simplicity of such a control program has led to widespread application in the field but to few attempts at controlled observations.

Washko and Zeissig [13] interpreted their results as being in essential agreement with those of the foregoing authors, but thought that additional oral medication with streptomycin and penicillin might be helpful. Dunlop and Strout [4] combined injection of the parent stock with a serological testing program (on a statewide basis) of the offspring. While dihydrostreptomycin was found to suppress hatching egg-transmission of PPLO, complete elimination was not obtained, and it was found advantageous to maintain hatches in small groups until proved free from PPLO infection. In general, it was possible, under commercial conditions, to raise birds apparently free from PPLO infection beyond the age of 24 weeks. The ultimate goal, according to Dunlop,[5] was to use only negative breeders. From these, 85,000 broilers have been produced with no evidence of air sac disease.

Critical evaluation of these reports would indicate that available treatments of the parent stock reduce egg-transmission of PPLO but do not achieve true prevention. Results may be influenced by the irregularity and unpredictability of PPLO egg-transmission, a phenomenon which was recently made the subject of experimental inquiry by Levine and his colleagues.[11] Furthermore, the criteria used for determining freedom from PPLO infection (frequently confined to clinical observations and the serum-plate test) are not sufficient unless accompanied by repeated cultural attempts. Even in the serum-plate test, the question of the existence of multiple avian PPLO has arisen from the observation of Adler *et al.*,[2] who differentiated a "pathogenic" group from a "nonpathogenic" group on the basis of cross agglutinability, ammonium sulfate flocculation, hemagglutinability, morphology, and reaction in the turkey sinus. Similar observations will be reported (section on Experimental Procedure), but final judgment as to the method of dealing with fundamentally different PPLO, especially nonpathogenic organisms, or with loss-variants, must be held in abeyance.

EXPERIMENTAL PROCEDURE

Experiments were conducted to study the influence on PPLO infection of ventilation, superinfection with infectious bronchitis, parenteral treatment of infected parent stock, and cultural and serological follow-up of offspring.

Spread of PPLO Under Different Conditions of Ventilation.—Two plywood cabinets (4 by 4 by 8 ft.) were constructed in cooperation with the University Department of Agricultural Engineering. One cabinet had an air exchange of 3 cu. ft. per minute, the other of only ½ cu. ft. per minute. Of 20, 4-week-old susceptible birds per cabinet, 10 were inoculated intratracheally with a broth culture of a patho-

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genic PPLO * and maintained for four weeks. The results were assessed on the basis of pre- and postinoculation geometric mean titers (GMT) to the PPLO tube-agglutination test [10] and histological examination of tracheas, lungs, and air sacs.[8]

The preinoculation GMT for all groups was 1.0. The postinoculation GMT at high ventilation for the inoculated group was 17.0; for the uninoculated group, 1.0; at low ventilation for the inoculated group, 37.0; for the uninoculated group, 4.3. Histological examination disclosed lesions indicative of PPLO infection in all groups except the uninoculated group (at high ventilation), thus confirming the serological findings.

It appeared that high ventilation decreased the spread of PPLO by contact. This finding is in harmony with field observations, where better ventilation often materially improved the clinical course of CRD. That this situation was peculiar to PPLO was suggested by a similar experiment with Newcastle disease, where increased ventilation intensified the spread of the disease.

Superinfection with Infectious Bronchitis.—Groups of approximately 10, 5-week-old birds were maintained in isolation and inoculated intratracheally with pathogenic PPLO,* infectious bronchitis virus (Massachusetts type [7]), and with both agents, either simultaneously or 11 days apart. In preliminary exeriments, it had been determined that this PPLO caused definite microscopic tracheal lesions between postinoculation days 12 and 16, followed by recovery. In the main experiment, the birds were killed on postinoculation day 28 and the tracheas prepared for histological examination. The birds inoculated with PPLO and infectious bronchitis alone, or with both agents simultaneously, were normal; the birds given infectious bronchitis virus 11 days after the PPLO inoculation had severe lesions which were indistinguishable from those described for field cases of CRD.[8]

These experiments furnished a clear-cut demonstration of the importance of multiple infections in the CRD picture, and suggested that the time of entry of the respective agents may cause further variations.

* Obtained from Dr. H. Adler, University of California, Berkeley.

Parenteral Treatment of Layers.—A flock of 140, 20-week-old White Rock birds, known to be infected with PPLO, was placed in an isolation house and one half the birds injected intramuscularly with 200 mg. of dihydrostreptomycin. All birds were tested serologically, and approximately 10 per cent were tested culturally,[9] at monthly intervals. Injection of dihydrostreptomycin failed to influence subsequent isolation of PPLO but decreased the GMT for the injected group. Positive isolations were obtained, at times, from birds both serologically positive and negative and from doubtful reactors.

Eggs were collected from both groups between postinjection days 9 and 23, and the resulting chickens were housed in isolation quarters and were tested by the same serological and cultural techniques used for the parent stock. In these experiments, the offspring from known PPLO-infected breeders remained free from the infection for periods up to 18 months, regardless of drug injection.

The over-all cultural results, obtained from January, 1956, to May, 1957, were of particular interest. In the infected flock, of 365 attempts to culture PPLO from tracheal swabs, 258 (70.7%) were positive; in the uninfected flock, of 858 attempts, a single suspicious strain was isolated which differed from other avian strains by its rapid breakdown of phenol red. It is noteworthy that, from the uninfected flock, neither pathogenic nor nonpathogenic strains of PPLO [2] were isolated.

Serological and Pathogenic Differences in PPLO Strains.—The presence of multiple avian PPLO [2] will complicate the picture. When the infected breeding flock was obtained, the first two cultural tests (about 1 month apart) yielded 26 hemagglutinating (HA+) strains in 34 attempts. Thereafter, none of the 157 positive isolates from 237 trials over a period of 11 months had this ability (HA–); other stock cultures, some of them maintained in the frozen state for years, and routine diagnostic isolates were HA+. Another flock of mature birds with a typical history of CRD was observed in the laboratory, with the result that 75 HA– isolates were obtained in 94 attempts.

To follow this puzzling phenomenon, homologous antigens and antiserums of HA+ and HA– strains were prepared in rabbits; it was found that HA+ antigens

usually reacted with HA– antiserums in the tube-agglutination test, but the HA– antigens failed to react with HA+ antiserums. Known susceptible birds were inoculated with the respective strains and subsequently cultured. In preliminary tests, the HA+ strains were reisolated for a period of about four weeks, then only HA– strains were obtained; whereas birds inoculated with HA– strains yielded only these strains. When inoculated into the anterior chambers of chicken eyes,[8] 18 of 22 HA+ strains caused severe microscopic lesions, but only three of 21 HA– strains caused mild lesions. Many of the postinoculation reactions could be read clinically.

These observations suggested the occurrence in nature of two types of avian PPLO: HA+, usually of high pathogenicity, as indicated by the eye test, and of homologous and heterologous agglutinability; and HA–, usually of low pathogenicity, as indicated by the eye test, and of limited agglutinability. According to available data, HA– strains represent serological loss-variants, which probably also exist in intermediate forms but are not found in known PPLO-negative flocks. Thus, they can not a priori be considered innocuous.

Field Tests

With the idea of establishing PPLO-free foundation stock, field tests were conducted on two large breeding farms. The blood samples were brought to the laboratory by messenger and tested by both the serum-plate and tube-agglutination procedures, the latter in one dilution of 1:12.5.

In flock A, the work was begun by obtaining about ten blood samples from birds (17 to 26 weeks old) in each of 30 units. Birds from the units showing relatively few reactors were subjected to a complete test and the PPLO-negative birds moved to a quarantine farm. The initial tests did not always prove good indicators of the distribution of reactors; for instance, one unit had 3 of 30 (10%) reactors on the initial test, but 594 of 1,173 (50.6%) on the complete test one week later. One difficulty was that the source of PPLO-free males was a unit which had 1,234 of 1,532 (80.5%) reactors. Eventually, 1,692 presumably PPLO-free birds were assembled on the quarantine farm but, on retests four and eight weeks later, the percentages of reactors were 39.9 and 84.9, respectively.

In another trial, a house containing 236 birds, 20 weeks old, was found to have 10 per cent reactors, which were removed. The remaining birds were then mated with PPLO-free males originating from a unit with 27 per cent reactors. When this "negative" flock was retested six weeks later, 50 per cent of the birds proved positive.

In flock B, each breeder was injected intramuscularly with 125 mg. of streptomycin and dihydrostreptomycin at monthly intervals from November, 1956, to May, 1957. The owner thought that the treatment improved hatchability and the percentage of saleable chicks. Eggs obtained one, two, or three weeks after injection of the parent stock were hatched separately. The resulting chickens were kept in adjacent units of less than 500 and blood tests were done in sample lots of 50 at approximately 10, 15, and 20 weeks of age. Many of the lots maintained this status on subsequent tests. One lot, with 2 per cent reactors at 10 and 15 weeks of age, had 48.4 per cent at 21 weeks. In another lot, the percentage of reactors varied from 0 to 70 to 4 up to 17 weeks of age. There was no definite trend in the percentage of reactors with respect to the interval from injection to egg collection, or to the number of injections given to the parent stock. The breeding performances of these lots with known serological histories will be awaited with interest.

On the whole, these two large-scale attempts at establishing PPLO-free breeding units, as judged by serological tests, have failed, particularly with flock A. It was believed that the frequent movements of birds and the lack of PPLO-free male units were contributing factors. The program used with flock B would permit the production of broiler chickens, the majority of which should be practically free from any liability toward CRD. This is a desirable feature, since it has been estimated by experienced broiler growers that the difference in the cost of production of CRD-affected lots versus lots not affected with CRD is 1.3 cents per pound.

Discussion and Summary

Proposed methods for the control of chronic respiratory disease (CRD) have as their ultimate aim the eradication of pleuropneumonia-like organism (PPLO) infection from the breeding stock.

The segregation of breeding stock into positive and negative birds, by PPLO serological tests, has not been found practical because the negative birds often rapidly become positive.

Sampling of young breeder units by serological tests is a useful procedure for recognizing the units with the lowest percentage of reactors. Promising units may be designated for parenteral treatment with streptomycin products.

Parenteral treatment is not standardized but should probably be held to a minimum number of injections. Antibiotic treatment for breaking the transovarian infective cycle of PPLO is not established as being essential.

Parenteral treatment of parent stock seems to produce a high percentage of chickens, with a low number of reactors to PPLO serological tests up to 10 weeks of age or more. The indications are that such treatments should be repeated every two weeks. The procedure is useful for the production of broiler chickens with a low liability toward CRD. Such chickens seem to have an advantage over infected birds in production cost of 1.3 cents per pound.

For the production of replacement stock, the hatches from promising parent stock units should be kept in relative isolation, in units not to exceed 500 birds. They should be moved as little as possible. The introduction of negative males from positive units into PPLO-free female units is potentially dangerous.

Promising units of prospective foundation stock should be subjected to bimonthly total blood tests, beginning with a 10 per cent sample test at the age of 8 to 10 weeks. In apparently PPLO-free units, it is advisable to take tracheal swabs of 10 or more birds for PPLO cultural tests.

Increased ventilation decreases the spread of PPLO under experimental conditions, and ameliorates the course of CRD under field conditions.

Superinfection with infectious bronchitis virus (and probably other agents) intensifies and prolongs PPLO infection and should be avoided.

Under natural conditions, avian PPLO occur as hemagglutinating (HA+) and nonhemagglutinating (HA–) types; the former usually of high pathogenicity, as indicated by the eye test, and of homologous and heterologous agglutinability, and the latter of low pathogenicity and poor agglutinability. The HA– type is considered a serological loss-variant. Neither type is found in known PPLO-free stock.

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Symposium on Chronic Respiratory Diseases of Poultry. V. Infectious Sinusitis of Turkeys

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PROBABLY the first to give an accurate description of infectious sinusitis was Dodd, in England in 1905.[13] Tyzzer,[53] in 1926, first recognized the disease in the United States.

HISTORY AND CAUSE

The term "infectious sinusitis" (IS) was suggested by Dickinson and Hinshaw,[12] in 1938, to differentiate the disease from inflammation of the sinuses of turkeys due to vitamin A deficiencies. Delaplane,[11] in 1949, reported that the causative agent of IS of turkeys was identical with that of chronic respiratory disease (CRD) of chickens. Nelson[40-43] was the first worker to isolate the causative agent of these two diseases, and he described it as being "coccobacilliform bodies" which would not grow in artificial mediums, but which could be cultivated in the fetal membranes of embryonating chicken eggs. He also observed that the agent was pleomorphic when cultivated in the embryonating egg or in tissue culture.[43] The causative agent was properly identified by Markham and Wong[35] as an organism similar to the pleuropneumonia group of organisms; they introduced the term "pleuropneumonia-like organism" (PPLO). They also were able to cultivate the organisms in artificial mediums containing 20 per cent equine serum.

Various workers since have confirmed the work of Markham and Wong and have characterized the agent as a pleomorphic organism (*Mycoplasma gallinarum*) which can not be cultivated on artificial mediums unless serum fractions, embryo extract, or ascitic fluid is added to the medium. The organism stains faintly with Gram's stain but stains well with Giemsa's stain.[1, 6, 24, 33, 35, 44] The agent grows well in the embryonating chicken egg and produces characteristic lesions consisting of hemorrhages of the skin of the embryo and of the amnionic and yolk membranes. The embryo is stunted in size and may have enlargements of the joints of the legs, wings, and articulations of the mandible.[5, 6, 10, 27]

The PPLO ferment certain carbohydrate mediums containing serum fractions. They produce acid, but no gas, in dextrose, maltose, and saccharose. Lactose and mannitol are not fermented.[20, 21] These biochemical characteristics are used in the identification of the organism.[18]

Mycoplasma gallinarum will pass through Berkefeld V, N, and Mandler 6 and 7 filters, but will not pass Berkefeld W, Selas 03, or Seitz EK filters. The agent is unable to survive a temperature of 45 C. for more than one hour, or 50 C. for more than 20 minutes. It remains viable for two years when frozen at −35 C., and the optimum pH of the organism is 6.1 through 8.6.[3] Electron photomicrographs have been made of the agent of IS, and it was found to be a filament between 50 to 60 mμ in width.[31, 56]

Recently, Fahey[16] has isolated a viral agent from turkeys which may have a role in the cause of IS of turkeys. This virus may act as a predisposing factor which may allow the PPLO to become more pathogenic. The relationship of this agent, as well as other viral agents, to IS has not been completely determined.

CHARACTERISTICS OF THE DISEASE

Host Range.—The host range of *M. gallinarum* includes turkeys, chickens, ducks, pheasants, guinea fowl, pigeons, partridges, peacocks, and the cotton rat.[55, 57] The Swiss albino mouse and the Norway rat are refractory.[31, 52]

Susceptibility of the Agent to Antibiotics.—Several authors have demonstrated that PPLO are sensitive to antibiotics.[20, 28, 58] The PPLO is susceptible to the broad-spectrum antibiotics, such as oxytetracycline, chlorotetracycline, and chloramphenicol, and also to erythromycin and streptomycin. Penicillin, neomycin, and the sulfonamides are not effective against PPLO.[4, 23, 28] Recently, antibiotic-resistant strains have been isolated from birds on antibiotic therapy.[15, 36, 48] The agent isolated by Fahey[17] is a true filterable virus and is not affected by the antibiotics known today.

Virulence.—There are many strains of PPLO and they appear to vary greatly in degree of virulence. Pathogenic and nonpathogenic strains have been isolated, and a method for identification of these strains has been devised.[2] Neurotropic strains have

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also been described in the literature.[7] Outbreaks of IS are usually prosodemic in character, and the incubation period may vary from nine to 31 days.[3] The morbidity in the natural disease may vary from 10 to 50 per cent, and the mortality may approach 25 per cent.[22, 50]

Modes of Transmission.—This disease is spread by direct contact with infected birds, and field observations also indicate that the disease is airborne. Investigators have demonstrated that, in apparently recovered flocks, as many as 20 per cent of the birds may remain permanent carriers.[32, 45, 55] Jerstad *et al.*,[29] in 1949, suggested that the IS agent might be egg-transmitted when they isolated it from fresh turkey eggs.[29] As a result of recent experiments, it has been clearly demonstrated that the causative agent of IS of turkeys is egg-transmitted,[28, 36, 37] which had been suspected on the basis of field observations in turkeys and egg-transmission studies in chickens.[9, 39, 54] The agent has been isolated from infertile eggs, dead embryos, pipped eggs, and hatched poults of infected hens.[37]

It has been postulated that, since the PPLO has been isolated from free-flying birds such as pheasants, partridges, and pigeons, these birds may have a role in the transmission of IS of turkeys and CRD of chickens. More investigation is needed before it can be conclusively determined how important free-flying birds are in the transmission of these diseases.

Clinical Signs.—The signs of the upper respiratory form of IS consist of a watery discharge from the nostrils and eyes followed by an enlargement of the infraorbital sinus.[34] Early in the course of the disease, the birds shake their heads and exhibit general signs of irritation of the mucous membranes. The swollen sinus may cause partial or complete obstruction of vision. The appetite remains good as long as the birds can see; however, weight gains are poor.[50]

The signs of the lower respiratory form of IS include dyspnea, moist tracheal râles, and cyanosis. If the birds are in egg production, it may decrease 10 to 30 per cent.

Pathological Changes.—The macroscopic lesions consist of gross enlargement of the infraorbital sinuses, with thickening and petechial hemorrhages of the membranes of the sinus. The lungs may be consolidated, and the air sacs are thickened and no longer transparent. A deposition of caseous material may occur on and in the air sacs.[24]

The microscopic lesions consist of catarrhal inflammation of the mucosa of the sinus early in the course of the disease. This is followed, in the later stages, by fibrosis and lymphofollicular infiltration of the submucosa of the sinus. The main lung lesions are those of bronchopneumonia[8] and the air sac lesions are those of lymphofollicular infiltration of the membranes.[30]

Differential Diagnosis.—Infectious sinusitis can not be diagnosed on the basis of clinical signs and lesions alone, as laboratory confirmation is necessary to separate the lower respiratory form of this disease from Newcastle disease, ornithosis, and aspergillosis. The lesions and signs of these diseases may mimic those of the lower respiratory form of IS. Confirmation of a diagnosis of IS is accomplished by isolation and cultivation of the agent *(M. gallinarum)*, identification by microscopy, and the biochemical characteristics of the agent.

CONTROL OF THE DISEASE

Breeding Flock Selection.—Turkey flocks with a history of having had IS should not be used as sources of breeding flocks, because the causative agent may be egg-transmitted.[26, 36, 37] At the present time, there is no way of predicting the degree of egg transmission. In some broods of poults from an infected breeding flock, only a few birds will develop clinical evidence of IS. In other broods from an apparently normal breeding flock, nearly all the poults will develop either the upper or the lower respiratory form of the disease. A recent survey in Minnesota indicated that the CRD complex was responsible for 11 per cent of the turkey death losses.[14] The incidence has increased since a 1951 survey.

Most hatcheries are accepting hatching eggs only from those breeding flocks which show no clinical signs of IS. Until a more definite program is available, the cooperation of all phases of the turkey industry is essential in the selection of replacement breeding flocks. Field observations suggest that the unapparent type of infection in breeding flocks may be responsible for some of the IS outbreaks.

Serological Tests.—There are many sero-

logical tests available to aid in the diagnosis of IS, and the rapid serum-plate agglutination test, the tube-agglutination test, the hemagglutination-inhibition test, and the freeze-agglutination test have been used. These tests have not been correlated well enough to determine which is the most satisfactory for turkeys.

A program was developed in 1956 and continued in 1957, in which a 10 per cent random sample of Minnesota turkey breeding flocks was selected and the serums tested by the rapid serum-plate agglutination test. The results in two groups of breeding flocks encouraged the expansion of the program in 1957.

One hatchery group (11 breeding flocks) was selected from flocks which have shown no clinical indication of IS over the past two years. A closed breeding flock program has been practiced in this organization for several years. The random sample testing of each flock was done at the beginning and end of the laying season. Tests for PPLO and ornithosis were negative for all flocks. Clinical IS has not occurred, and retests to date on the progeny that will be used for the breeding flocks this season (1958) have been negative.

The second group involved 12 breeding flocks from a hatchery that had a number of attacks of IS during the season. These were selected because it was impossible to select all the replacement flocks from flocks that had no clinical history of IS during the growing period. All breeder flocks that had a clinical history of IS were treated with monthly injections of streptomycin, and continuously fed a tetracycline in the feed. The random sample testing of these flocks at the beginning and end of the laying season revealed that 60 per cent of the flocks were positive to the PPLO test. Clinical IS has occurred in the progeny hatched from these flocks this season. The replacement flocks for this season have not been tested.

Antibiotics and Chemotherapeutic Agents.—The tetracyclines, as well as erythromycin and streptomycin, are effective in controlling mortality and treating infected sinuses.[20, 25, 28, 46] Penicillin, neomycin, and sulfonamides appear to have little effect on the agent.[4, 23, 28] Silver nitrate and argyrol have been injected directly into the sinus to cauterize the secreting sinal tissue and reduce the enlargement of the infraorbital sinus.[34, 38, 53] The use of antibiotics in breeding flocks has been advocated to reduce the hazard of egg-transmission of PPLO. Osborn and Pomeroy[48] found that prolonged high-level antibiotic therapy of turkey hens did not completely block egg transmission. The occurrence of antibiotic-resistant PPLO following antibiotic therapy may be a problem in the treatment of the disease.[15, 36]

General Management Practices.—When the disease occurs on a turkey farm that has multiple broods, it has been observed that it eventually is carried to the other groups. The infected flocks may develop permanent carriers which serve as a source of the infection to other flocks. The resistance developed by older birds that have had the disease does not appear to be of protective antibody type. It seems to be autarcetic in nature, as little protective antibody can be demonstrated in the serum of actively infected or recovered turkeys.[49] It appears that the only method of eliminating the infection is complete depopulation and cleaning and disinfection of the equipment. Quaternary ammonium compounds, phenolic compounds, and cresolic solutions were effective in destroying the organism[47] in *in vitro* studies.

SUMMARY

1) Infectious sinusitis (IS) continues to be an important problem of the turkey industry. Little progress has been made in reducing the incidence of the disease.

2) Advancements in the knowledge of the etiological agent, modes of transmission, and control have resulted from the research programs coordinated on a national level.

3) The ultimate development of a control program for IS awaits further study on the effectiveness of the serological tests in detecting carrier flocks.

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A Cannulated Thiry-Vella Fistula in the Chicken

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THE SURGICAL PROCEDURE to be described was devised to serve as a companion system to *in vitro* preparations of surviving intestine in studies of absorption from chicken intestine and represents, to our knowledge, the first description of such a surgical preparation using the chicken. The method used in the living chicken is a variation of an old technique (Thiry-Vella fistula). Thiry resected a portion of a dog's intestine, brought one end of the intestine through the abdominal wall and closed the other end, leaving the resected loop, with blood supply intact, lying freely in the abdominal cavity. Vella improved this technique by exteriorizing both ends of the loop. In the work reported here, the primary problem of adapting the fistula for easy introduction and removal of test solutions into and from the fistula was solved by the use of cannulas.

OPERATION AND DISCUSSION

For a discussion of general surgical technique and anatomical data on the chicken, the reader is referred to general texts.[1,2,7] The details of the operation follow.

After withholding food for 12 hours to empty the intestine, the bird is fastened on an operating board, on its left side, with its right leg pulled forward to expose the right lateral abdominal wall. A 26-gauge needle is used to inject the anesthetic, an aqueous solution of sodium pentobarbital (50 mg./ml.), initially into the brachial vein (20 mg./lb. of body wt., for 3 minutes). More is given intramuscularly as needed to maintain anesthesia. For a 3-lb. bird, a total dose of 150 mg. is usually given during the three-hour operation.

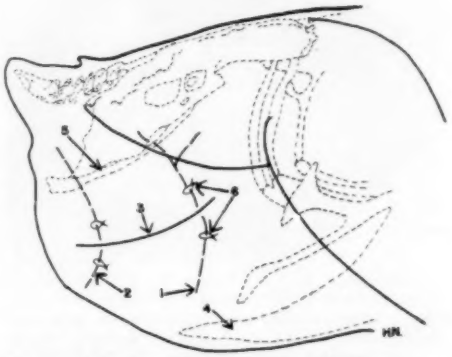

Fig. 1—Operative site (lateral abdominal wall) for cannulated fistula in the chicken: caphalad subcutaneous blood vessel (1); caudad subcutaneous blood vessel (2); incision (3); sternum (4); pubis (5); ligatures (6).

The feathers are cleared from the surgical area, which is then swabbed with tincture of iodine.

After ligating the two subcutaneous veins which run dorsoventrally in this

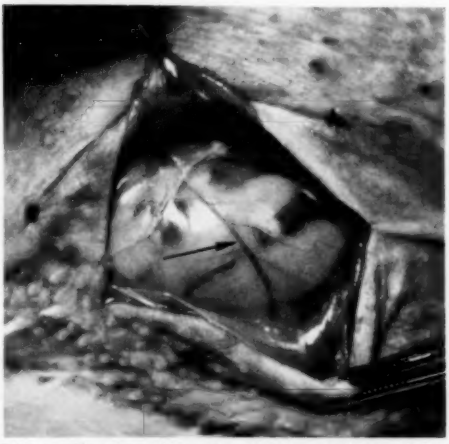

Fig. 2—Usual position of artery (arrow) associated with abdominal fat layer.

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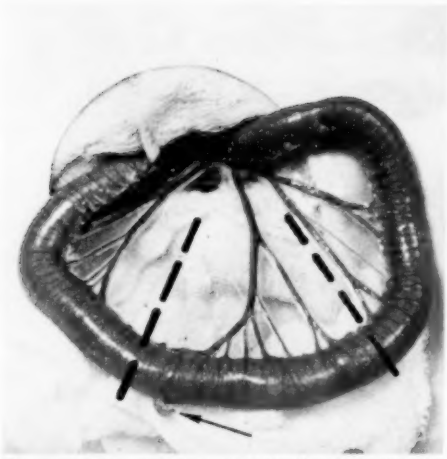

Fig. 3—Area of the small intestine in which the fistulas were formed, showing the yolk stalk (arrow) and location of the incisions (dotted lines).

area (fig. 1), the skin incision is started approximately 1 cm. from the last rib and continued caudally approximately 4 cm., through the abdominal wall and right abdominal air sac. In younger birds, the transverse abdominal muscle is firmly attached to the peritoneum and these two are usually penetrated as one. In birds over 6 months old, there is considerable abdominal fat between the transverse abdominal muscle and the peritoneum, which contains a large artery (fig. 2) that must be located and ligated before the fat layer is penetrated.

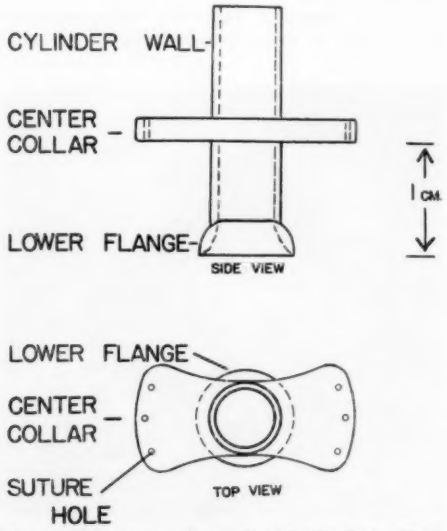

Fig. 4—Nylon cannula used with the Thiry-Vella fistula.

The margins of the wound are packed with gauze saturated with warm Ringer's solution to keep all exposed tissues moist until the wound is closed. With the small intestine visible, a hooked probe is used to withdraw the segment of small intestine which has the yolk stalk attached (fig. 3). The yolk stalk area in the chicken corresponds to the ileum of the mammalian intestine.

A portion of intestine, 5 to 7 cm. long and including the first radial mesenteric blood vessels on either side of the yolk stalk, is then prepared for resection. The mesenteric blood vessels immediately orad and caudad to the points where the intestine is to be resected are ligated with 4-0 medium chromic gut suture, and the intestine is severed between the ligatures, with the incision continuing well into the supporting mesentery between the radiating blood vessels.

A nylon cannula (fig. 4), devised to be fastened to the intestine and extend outside of the body wall to facilitate the introduction and removal of solutions from the isolated loop of intestine, is inserted into each end of the resected loop. The end of the intestine is slipped over the lower flange (fig. 5) and fastened in place in the angle formed by the lower flange and the cylinder wall by using a purse-string suture of 5-0 medium chromic gut. A second purse-string suture is placed approximately 3 mm. below the first suture line to invert the intestine (fig. 6) over the first suture. The cannulated intestinal loop is then packed in gauze which is saturated with Ringer's solution.

The continuity of the interrupted intestine is re-established with a side-to-side anastomosis (fig. 6), after the transected extremities are closed by inverting the ends with a purse-string suture (4-0 medium chromic gut), and a second concentric reinforcing row of sutures is placed on the stumps. The ends of the intestine are brought into apposition so that they are isoperistaltic and are maintained in that position with temporary guy sutures while incisions, approximately 2 cm. long, are made along the antimesenteric border of each end of the intestine. These intestinal openings are then joined with a Connell

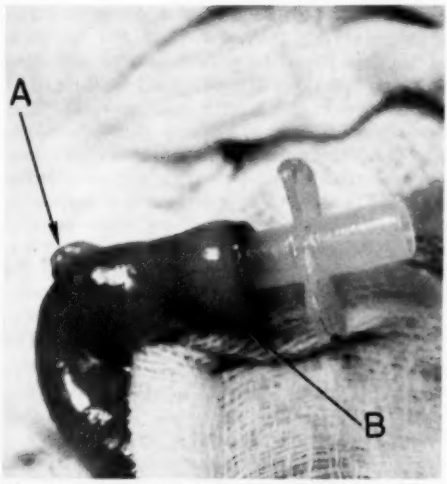

Fig. 5—Cannulation of the resected intestinal loop, showing (A) the yolk stalk, and (B) the intestine in position over the lower flange of the cannula.

suture, using No. 5-0 medium chromic gut with a ½-round needle. The inversion effected by the Connell suture must be complete. The rent in the mesentery is repaired, using medium chromic gut and an interrupted mattress suture, and the rejoined intestine is replaced in the body cavity.

All packing is removed and the cannulated fistula is replaced in the body cavity with the orifices of the cannula protruding through each end of the incision. The peritoneum and muscle layers are closed, using 3-0 medium chromic gut and an interrupted mattress suture, so that they lie between the flange and the center collar of the cannulas. The cannulas are firmly sutured to the muscle layers, the holes in the center collar of the cannulas being used as a means of attachment (fig. 7). The skin is closed over the center collar with interrupted cotton mattress sutures (fig. 8).

The only postoperative treatment necessary is a daily flushing of the fistula with warm Ringer's solution to keep the intestinal loop free of debris.

RESULTS

Once the surgical technique was mastered, approximately 75 per cent of the 110 chickens operated upon were satisfactory for experimental use in absorption studies (to be described later). Some of

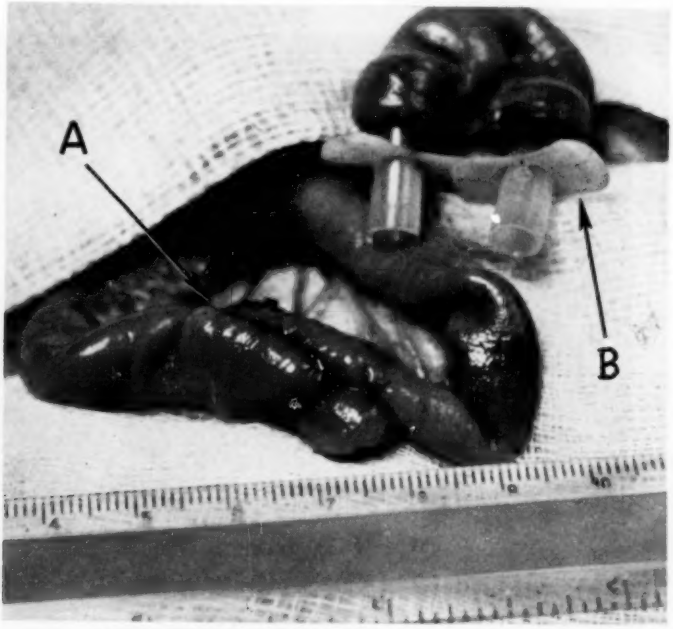

Fig. 6—Completed side-to-side anastomosis; (A) anastomosis and (B) cannulated intestinal loop.

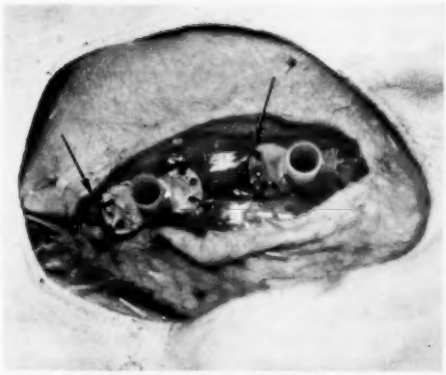

Fig. 7—The cannulas fastened in place by sutures through the underlying muscle layers and the holes in the center collar of the cannulas (arrows).

the other birds could not be used experimentally, either because a cannula came loose or the fistula became plugged. Some birds survived as long as six months postoperatively, before being killed, with no apparent ill effects.

Perhaps the most significant criticism of any surgically prepared specimen is that the subject no longer represents a normal physiological system. Histological studies of the intestine from the fistulas of chickens killed three to five months postoperatively showed that the portion of the intestinal loop free of the cannulas was morphologically normal, while the portion in contact with the cannulas had undergone a papyrus-type necrosis without liquefaction or caseous degeneration, leaving that area in a dry, nonabsorptive state. To ascertain whether the histologically normal portions were also physiologically normal, drugs with varying effects were given intravenously and the response of the loop tissue recorded with a standard kymograph system.[3,4,8] Tracings were made prior to and after the drugs were injected into the brachial vein. In the 2 chickens tested in this way, histamine (2.5 mg./kg. body wt.) and acetylcholine (14 mg./kg. body wt.) caused a marked increase in the number of intestinal contractions; atropine sulfate (0.3 mg./kg. body wt.) blocked the effect of acetylcholine; and epinephrine (10 to 15 mg./kg. body wt.) stopped intestinal motions so that none could be recorded on the kymograph.[5,9] It was concluded that the intestinal tissue exhibited a normal physiological response to the drugs used and that there was indication, but not proof, of the normal functioning of the

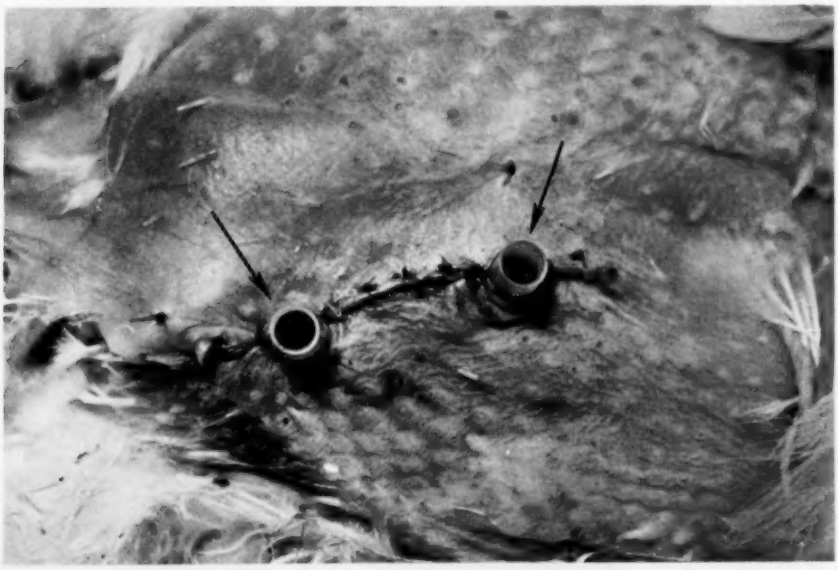

Fig. 8—Completed Thiry-Vella fistula, with the cannulas (arrows) extending through the lateral abdominal wall.

extrinsic nerve supply to the intestinal loop.

An aggregate material of rather soft consistency was found in the fistula postoperatively. Its source was not established, but it was possibly a normal material from a natural process; rapid sloughing of the cells at the tips of the villi and copious secretion of mucus by the intestine has been reported.[6] More probably, the pressure of the cannulas stimulated secretion by the intestine which led to the formation of the aggregate. A similar material has been observed by other investigators.[3,4] The fistulas occasionally became plugged with the hardened aggregate material and the birds had to be discarded, but daily flushing of the loop with warm Ringer's solution minimized this problem and obviated the need of cauterization, the method used by Streeten and Williams.[8]

SUMMARY

A modification of the Thiry-Vella fistula for use in the study of absorption from the small intestine of the chicken is described. The ends of the isolated loop of intestine are exteriorized by means of nylon cannulas. The interrupted intestine is anastomosed. Approximately 75 per cent of the 110 prepared specimens functioned satisfactorily. Some birds survived for six months with no apparent ill effects.

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Flocculation Tests for Pullorum Disease

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PRESENT METHODS of performing the tube-agglutination test for pullorum disease are time consuming. Publications of Bushnell,[1] Bushnell and Brandly,[2,3] Bushnell and Hudson,[4,5] Galloway and Holtman,[7] and Reid[12] reported discrepancies among all techniques now being used. To reduce discrepancies obtained in various laboratories, attempts have been made to develop standard antigens and methods. The Animal Disease and Parasite Research Division of the Agricultural Research Service of the U.S. Department of Agriculture has recommended a "standard" antigen and standard procedure for both the plate- and tube-agglutination tests for pullorum disease in fowl.

Kline[11] reported a microscopic slide test for syphilis in which he used an antigen emulsion composed of cholesterol coated with the acetone-insoluble fraction of beef heart extract; later, he used cardiolipin and lecithin. Harris *et al.*[8] developed a macroflocculation test for syphilis, using cholesterol coated with cardiolipin and lecithin as an antigen. These tests for syphilis have been reproducible and have improved sensitivity and specificity.

Hunter and Colbert[9] described a method of preparing an antigen emulsion by coating cholesterol with dialyzed ectoantigen extract of Brucella. Hunter and Colbert[10] stated that the sensitivity and specificity of the antigen emulsion could be adjusted to conform to the standard antigen. To overcome variability of the antigen and inconsistencies in performing the agglutination test for pullorum, the procedure of Hunter and Colbert[9] was used. *Salmonella pullorum* "regular" strain* was used in preparing the antigen.

MATERIALS AND METHODS

Preparation of Ectoantigen Extract of Salmonella Pullorum—Preparation of the antigen extract consisted of growing *S. pullorum* on thiosulfate-glycerol medium in Blake bottles for 72 hours at 37 C., and washing off the culture with 0.85 per cent saline solution containing 0.25 per cent phenol (additional phenol was added to the pooled suspension to bring the final concentration to 2.0%). The suspension was allowed to stand for seven days at approximately 8 C. and was then dialyzed in running water for 24 hours, or until free of phenol.[10] After centrifugation at 5,000 r.p.m. for 30 minutes, the supernatant fluid was removed, and the sedimented organisms discarded. The supernatant fluid was made isotonic by adding NaCl, and merthiolate was added to a final concentration of 1:10,000. The pullorum ectoantigen extract was kept either in a freezer at −18 C. or in a refrigerator at 8 C. until used.

Lecithin.—Lecithin (ex-beef) in absolute alcohol† was further diluted in absolute ethyl alcohol so that the amount of lecithin was 10 mg. per milliliter, or a 1.0 per cent solution.

Cholesterol Solution.—The cholesterol solution was 1.0 per cent cholesterol (Pfanstiehl; ash-free, precipitated from alcohol for the Kline test) in absolute ethyl alcohol.

Other Reagents.—Other reagents used were: distilled water (approx. pH 6.0), 0.85 and 1.0 per cent solutions of NaCl (Mallinckrodt's, analytical reagent (AR) grade), and Eagle's[6] buffered saline solution (pH 7.4).

Apparatus.—Equipment necessary to perform these tests is found in almost any laboratory where serological tests for syphilis are performed.

Preparation of Serums.—The blood serums to be examined were separated from the clots by centrifuging and pipetting or decanting. The serums were then heated for 15 minutes at 56 C. Serums held over a day for re-examination should be reheated for five minutes.

MICROSCOPIC SLIDE TEST

Preparation of Antigen Emulsion.—The antigen emulsion should be prepared as follows:

1) Pipette 0.85 ml. of distilled water (about pH 6.0) to the bottom of a 30-ml. round, flat-bottomed, glass-stoppered bottle.

2) While rotating the bottle, add 1.0 ml. of cholesterol solution, drop by drop. Continue rotating for 20 seconds.

3) Add 0.1 to 0.2 ml. of 1.0 per cent lecithin solution and shake vigorously for 15 seconds. The exact amount of lecithin to add must be determined by standardization according to methods to be discussed under "Method of Standardizing Antigen." Titration is necessary only for each new lot of lecithin or pullorum extract, or both.

Contribution No. 323 from the Department of Bacteriology, Kansas Agricultural Experiment Station, Manhattan. Professor Foltz is bacteriologist, Kansas Agricultural Experiment Station, Manhattan.

* Obtained from Professor L. E. Erwin, Kansas State College, Manhattan.

† The lecithin solution was supplied by Dr. Sol Rosenberg, East Orange, N.J.

4) Add 0.1 to 0.25 ml. of dialyzed, cell-free pullorum ectoantigen and agitate vigorously by repeatedly striking the bottom of the bottle against the palm of the hand for one minute. The amount of pullorum ectoantigen extract to be added must be determined for each lot of extract (to be discussed under "Method of Standardizing Antigen").

5) Finally, add 2.5 ml. of Eagle's buffered saline solution and shake gently for 30 seconds.

The antigen emulsion is usable immediately and, if refrigerated, may be used anytime within 24 hours.

Procedure of Performing Microscopic Slide Test.—The microscopic slide test should be performed as follows:

1) Pipette 0.05 ml. of serum or serum dilutions into paraffin or permanent ring glass slides.

2) Add 1 drop of antigen emulsion to each serum, using an 18-gauge blunt-pointed needle held perpendicularly. This needle should deliver 80 to 85 drops of antigen emulsion per milliliter.

3) Rotate the slides for four minutes on a mechanical rotator at a speed of 180 r.p.m.

4) Read immediately at a magnification of x 100.

5) A positive and a negative serum and a saline control should be included. All negative and saline controls should show no aggregation of crystals.

6) The reactions are recorded as follows: positive = P (+), presence of large and medium-sized masses of crystals; weakly positive = WP (±), presence of small aggregates; negative = N (−), no aggregation of crystals.

If positive results are obtained with 0.05 ml. of serum, it will be necessary to make dilutions of the serum in 0.85 per cent saline solution. It is recommended that twofold dilutions be made from 1:10 to 1:640, using 0.05 ml. of each dilution in performing the test. The titer of the serum obtained by the microscopic slide test will not be comparable to that obtained by the tube-flocculation test or by the standard tube-agglutination test, because the serum dilutions are not identical but are within the same relative range.

MACROSCOPIC TUBE-FLOCCULATION TEST

Preparation of Diluted Antigen Emulsion.—The antigen emulsion, as prepared for the microscopic slide test, is diluted 1:4 with 1.0 per cent sodium chloride solution. This diluted antigen must stand for at least five minutes before being used, to become stabilized; if refrigerated, it may be used within 24 hours.

Procedure of Performing the Macroscopic Tube Test.—The macroscopic tube-agglutination test should be performed as follows:

1) Make twofold dilutions, ranging from 1:10 to 1:640, of the heated serum in 0.85 per cent saline solution. The volume of each dilution in the tube should be 0.5 ml.

2) Add 0.5 ml. of diluted antigen emulsion to each tube. The final dilution will then range from 1:20 to 1:1,280.

3) Shake tubes on Kahn shaker for five minutes.

4) Centrifuge all tubes for ten minutes at 2,000 r.p.m. (International centrifuge size No. 2; No. 925 head, 4 place bronze).

5) Shake all tubes on Kahn shaker for exactly one minute.

6) Read the tubes immediately by holding them in front of a reading lamp against a black background. Record the results as follows: positive = P (+), presence of large aggregates and clear supernatant fluid; weakly positive = WP (±), crystals aggregated but supernatant fluid cloudy; negative = N (−), no aggregation of crystals but cloudy supernatant fluid which gives a swirl similar to that seen in a mixture of blood in saline solution.

METHOD OF STANDARDIZING ANTIGEN

In the preparation of antigen for various flocculation tests for syphilis, serologists have found that experiments must be conducted to determine the optimum ratio of reagents to be used. Antigens must be prepared using various ratios and concentrations of cardiolipin, lecithin, and cholesterol, so that the antigen will give the same sensitivity and specificity as the standard.[30] The same is true in standardizing the pullorum antigen, the only difference being that pullorum extract is substituted for cardiolipin. The standardization was done by making 32 antigen emulsions by mixing each of 0.05, 0.1, 0.15, 0.2, and 0.25 ml. of pullorum extract with 0.0, 0.05, 0.1, 0.15, 0.2, 0.25, and 0.3 ml., respectively, of 1.0 per cent lecithin, excluding the mixtures of 0.20, 0.25, and 0.30 ml. of 1.0 per cent lecithin, with 0.25 ml. of pullorum extract. The standardization was carried out on heated serums because the final tests were to be on heated serums.

The microscopic slide-flocculation tests were run on at least nine serums of various reactivity with each of the 32 antigen emulsions. Thus, the ratio of pullorum extract to lecithin giving the same sensitivity and specificity as the standard tube antigen was determined. Once this ratio was determined for a particular lot of pullorum extract and lecithin (1.0%), it could be used until the lot was exhausted. In other words, it is necessary to standardize each lot of pullorum extract and lecithin (1.0%) only once.

The final ratio used to compare with the standard tube-agglutination test was found to be 0.15 ml. of lecithin (1.0%) and 0.15 ml. of pullorum extract.

RESULTS

Comparisons of results of the routine tube-agglutination test with the microscopic slide-flocculation test are shown (tables 1, 2). These tables represent data from 445 serums (turkeys, chickens, hu-

man type "O" as negative control, serums of goats inoculated with *S. pullorum*, and serum of a goat inoculated with *Salmonella typhosa*).

If the results in tables 1 and 2 are compared, the number of 4+ positive tubes is increased in a majority of the cases when the serums are heated. The positive reactions could be easily read. The end points were sharp and definite and did not give any questionable flocculation.

The results obtained by a group of students using the microscopic slide-flocculation test, on both heated and unheated serums, were similar to the results obtained with serums from the following sources: the 8 goats inoculated with *S. pullorum*, the goat inoculated with *S. typhosa*, and negative human "O" serum.

These results demonstrated that independent inexperienced workers could duplicate the results on the same serums. The comparison of the tests performed by these workers also showed that the heated serums gave more distinct results and sharper end points than nonheated serums. However, the titers were slightly variable.

Anti-*S. typhosa* serum gave a positive reaction with the *S. pullorum* extract, since it contains the same somatic antigen components, IX and XII.

DISCUSSION

The use of cholesterol crystals coated with cardiolipin and lecithin as an antigen for serological tests for syphilis has shown that the tests can be standardized, are reproducible, and have acceptable sensitivity and specificity. No other laboratory procedure has so high a level of reproducibility, from laboratory to laboratory, as this serological test for syphilis. The use of cholesterol crystals coated with a dialyzed, cell-free ectoantigen extract of *S. pullorum* should also produce an antigen in which the sensitivity and specificity

TABLE 1—Comparison of the Tube-Agglutination Test with the Microscopic Slide- and Macroscopic Tube-Flocculation Tests for Pullorum Disease, Using 445 Heated Serums

Test	Dilutions	Goats inoculated with *S. pullorum* * (No.) 1	2	3	4	5	6	7	8	Goat inoculated with *S. typhosa*	Human "O" negative	Turkey	Turkey	Chicken serums (63)	Turkey serums (370)
Tube-agglutination	10	4+	4+	4+	3+	4+	2+	2+	4+	3+	—	3+	—	—	—
	20	4+	4+	3+	3+	4+	2+	2+	4+	3+	—	3+	—	—	—
	40	4+	4+	3+	3+	4+	2+	2+	4+	3+	—	3+	—	—	—
	80	4+	4+	3+	3+	3+	2+	1+	3+	2+	—	2+	—	—	—
	160	4+	3+	3+	3+	2+	2+	—	3+	1+	—	—	—	—	—
	320	2+	3+	2+	2+	2+	2+	—	2+	—	—	—	—	—	—
	640	—	3+	1+	1+	1+	2+	—	—	—	—	—	—	—	—
	1280	—	2+	—	—	—	—	—	—	—	—	—	—	—	—
	2560	—	1+	—	—	—	—	—	—	—	—	—	—	—	—
Tube-flocculation	10	+	+	+	+	+	+	+	+	+	—	—	+	—	—
	20	+	+	+	+	+	+	+	+	+	—	—	+	—	—
	40	+	+	+	+	+	+	±	+	+	—	—	+	—	—
	80	±	+	+	+	+	+	—	+	±	—	—	±	—	—
	160	—	+	+	+	±	+	—	—	—	—	—	—	—	—
	320	—	+	±	+	±	±	—	—	—	—	—	—	—	—
	640	—	±	—	±	—	—	—	—	—	—	—	—	—	—
	1280	—	—	—	±	—	—	—	—	—	—	—	—	—	—
	2560	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Microscopic slide-flocculation	1.25	+	+	+	+	+	+	+	+	+	—	+	+	—	—
	12.5	+	+	+	+	+	+	±	±	±	—	—	+	—	—
	25	+	+	+	+	+	+	±	±	—	—	—	+	—	—
	50	±	+	+	+	+	+	—	±	—	—	—	+	—	—
	100	—	+	+	+	±	+	—	±	—	—	—	—	—	—
	200	—	±	±	+	—	±	—	—	—	—	—	—	—	—
	400	—	—	—	±	—	—	—	—	—	—	—	—	—	—
	800	—	—	—	±	—	—	—	—	—	—	—	—	—	—
	1600	—	—	—	—	—	—	—	—	—	—	—	—	—	—

* All goats were inoculated subcutaneously, twice yearly for a minimim of five years, with 18 inoculations of 5.0 ml. of a suspension comparable to the nephelometer tube three. Eight goats were inoculated with the "regular" strains of *S. pullorum* and 1 with *S. typhosa* (antigens IX, XII (no Vi):d). The number of positive serums was limited. The turkey being inoculated with *S. pullorum* died during the period of inoculation, so further work along this line was abandoned.

4+ = large clumps with clear supernatant fluid; 3+ = medium clumps with clear supernatant fluid; 2+ = small clumps with cloudy supernatant fluid; 1+ = small clumps with individual particles and cloudy supernatant fluid; + = positive; ± = weakly positive; — = negative, no flocculation.

TABLE 2—Comparison of the Tube-Agglutination Test with the Microscopic Slide- and Macroscopic Tube-Flocculation Tests for Pullorum Disease, Using 445 Unheated Serums

Test	Dilutions	Goats inoculated with *S. pullorum* * (No.) 1	2	3	4	5	6	7	8	Goat inoculated with *S. typhosa*	Human "O" negative	Turkey	Turkey	Chicken serums (63)	Turkey serums (370)
Tube-agglutination	10	4+	4+	3+	3+	4+	2+	4+	3+	3+	—	3+	2+	—	—
	20	4+	4+	3+	3+	4+	2+	4+	3+	3+	—	3+	—	—	—
	40	4+	3+	3+	3+	4+	2+	4+	3+	3+	—	3+	—	—	—
	80	3+	3+	2+	2+	3+	2+	4+	2+	2+	—	2+	—	—	—
	160	2+	2+	2+	2+	2+	2+	1+	1+	2+	—	—	—	—	—
	320	1+	2+	2+	2+	2+	—	—	—	1+	—	—	—	—	—
	640	1+	1+	1+	2+	1+	—	—	—	1+	—	—	—	—	—
	1280	—	1+	—	2+	1+	—	—	—	—	—	—	—	—	—
	2560	—	—	—	1+	—	—	—	—	—	—	—	—	—	—
Tube-flocculation	10	+	+	+	+	+	+	+	+	+	—	±	+	—	—
	20	+	+	+	+	+	+	+	+	+	—	±	+	—	—
	40	+	+	+	+	+	+	+	+	+	—	±	±	—	—
	80	±	+	+	+	+	+	+	+	+	—	—	—	—	—
	160	—	+	+	+	+	+	+	+	+	—	—	—	—	—
	320	—	+	+	+	+	+	±	±	±	—	—	—	—	—
	640	—	±	±	±	+	+	—	—	—	—	—	—	—	—
	1280	—	±	—	—	±	±	—	—	—	—	—	—	—	—
	2560	—	—	—	—	—	±	—	—	—	—	—	—	—	—
Microscopic slide-flocculation	1.25	+	+	+	+	+	+	+	+	+	—	+	+	—	—
	12.5	±	+	+	+	+	+	+	+	±	—	±	—	—	—
	25	±	+	+	+	+	+	±	+	±	—	±	—	—	—
	50	—	+	+	+	+	+	±	+	±	—	—	—	—	—
	100	—	±	+	+	+	+	—	±	±	—	—	—	—	—
	200	—	±	±	+	±	+	—	±	—	—	—	—	—	—
	400	—	—	±	±	±	+	—	—	—	—	—	—	—	—
	800	—	—	—	±	—	±	—	—	—	—	—	—	—	—
	1600	—	—	—	—	—	±	—	—	—	—	—	—	—	—

* All goats were inoculated subcutaneously, twice yearly for a minimum of five years, with 18 inoculations of 5.0 ml. of a suspension comparable to the nephelometer tube three. Eight goats were inoculated with the "regular" strains of *S. pullorum* and 1 with *S. typhosa* (antigens IX, XII (no Vi):d). The number of positive serums was limited. The turkey being inoculated with *S. pullorum* died during the period of inoculation, so further work along this line was abandoned.

4+ = large clumps with clear supernatant fluid; 3+ = medium clumps with clear supernatant fluid; 2+ = small clumps with cloudy supernatant fluid; 1+ = small clumps with individual particles and cloudy supernatant fluid; + = positive; ± = weakly positive; — = negative, no flocculation.

can be established, thereby producing a standard antigen. The use of this antigen in performing the flocculation tests for pullorum disease will give uniform results that are reproducible in different laboratories. Furthermore, the test can be run in a shorter period than the 24 hours required for the *S. pullorum* tube-agglutination test. The tests are much easier to read than the agglutination tests, and mistakes in interpretation of the titer should be decreased.

The pullorum extract used in this work was satisfactory but, if work is to continue on this problem, an effort should be made to separate a more specific fraction or fractions of the extract. If these tests were to be used, especially the microscopic slide-flocculation test, it probably would be necessary to re-evaluate the significance of titers with proved pullorum-infected birds to establish a "significant standard titer" for diseased fowl.

SUMMARY

1) A method of producing a cholesterol-lecithin extract antigen of *Salmonella pullorum* that can be used to perform a microscopic slide-flocculation test and a macroscopic tube-flocculation test has been described.

2) The sensitivity and specificity of the antigen can be adjusted to conform to the standard *S. pullorum* tube-agglutination test.

3) A method of standardizing the antigen emulsion is described.

4) The test can be performed rapidly, and the results are clear-cut and easily read.

5) The test results can be readily reproduced.

6) Heating the serums at 56 C. for 15 minutes gave a better distribution of the floccules and, consequently, gave more accuracy in the interpretation of the results.

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Immune Response of Chickens to *Beta*-Propiolactone-Killed Newcastle Disease Vaccines

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IT IS a basic immunological concept that the selection and inactivation of appropriate viral strains will, in a large measure, determine the antigenicity of inactivated virus vaccines. In the search for a viral inactivator which would guarantee a high degree of antigenicity while not being deleterious to plasma protein, *beta*-propiolactone (BPL) has been found to be highly effective. It is the purpose of this paper to present the results of studies of the comparative immunizing potency of vaccines prepared from three strains of Newcastle disease virus (NDV) inactivated by BPL.

Hartman *et al.*[4] and Mangun *et al.*[16] have found BPL to be one of the most promising virucidal agents of 140 compounds screened. The former authors reported that BPL, in concentrations of below 1,500 mg. per liter, effectively inactivated a 1 per cent suspension of NDV-infected brain tissue in the presence of 95 per cent plasma and whole blood. Smolens and Stokes[21] treated serums heavily infected with a virus (bacteriophage T4) by ultraviolet irradiation, followed by BPL in concentrations as low as 1.5 Gm. per liter. Under these conditions, the authors did not observe any changes in the serum protein as determined by electrophoretic analysis.

LoGrippo *et al.*[13] studied the concentration of BPL required to inactivate viruses, fungi, bacteria, and desiccated spores, and reported that BPL satisfactorily inactivated these agents at concentrations ranging from 0.05 to 0.7 per cent. LoGrippo and Hartman[14, 15] described a simple and rapid procedure for the preparation of BPL-inactivated virus vaccines. These authors found that BPL is capable of inactivating viruses within ten to 15 minutes at 37 C. They succeeded in demonstrating a higher degree of antigenicity in viral vaccines (the MM-strain of murine encephalomyelitis, eastern equine encephalomyelitis, and rabies viruses) inactivated with BPL than in those killed by formalin and phenol. Virus inactivation was found by the same authors to occur within a pH range of 5.0 to 9.0, permitting a choice of optimum pH.

Mack and Chotisen[17] used BPL as an inactivating agent in a Newcastle disease vaccine and, after using it on 43 birds, were able to demonstrate 100 per cent resistance to challenge 16 days later. These workers reported that 97.4 per cent of the birds in an unvaccinated control group succumbed to challenge.

Burmester[2] succeeded in providing a passive immunity for progeny of hens vaccinated with formalin or BPL-inactivated, visceral lymphomatosis vaccines. This vaccine was considered by the author to be immunogenic, although the immunity was found to be less than that obtained by the use of a live virus vaccine. A recent report[20] indicates that BPL has been used successfully for virucidal purposes in the preparation of rabies vaccine for use in man.

Huddleson[8] observed that Brucella cells of three colonial types were rapidly inactivated by BPL in final concentrations of 0.10 to 0.15 per cent. Cells of one type of *Brucella suis* inactivated with BPL produced a high degree of immunity in guinea pigs against infection with *Br. suis*, provided that two injections were given at intervals of ten days.

Kelly[10] and Kelly and Hartman[12] studied the biological effects of BPL and observed that it had a low toxicity index. Kelly *et al.*[13] found that daily transfusions of BPL-treated plasma did not produce detectable effects in men or dogs. Electrocardiograms, hemograms, body weight, and liver and kidney functions were not adversely affected. Kelly and Hartman[11] have found that BPL was suitable for sterilization of blood and plasma. The intravenous mouse l.d.$_{50}$ was found by these authors to represent a value of 406 mg. per kilogram, or one-hundredth that of nitrogen mustard, with which the effectiveness of BPL was compared. Hartman and Kelly[5] investigated tissue toxemia of BPL and its degradation products. They injected the degraded form of BPL intravenously and succeeded in producing a marked, congestive necrosis of the liver parenchyma and degeneration of the tubules of the kidneys in rabbits and dogs.

MATERIALS AND METHODS

Experimental Birds.—The majority of chickens used in this study were standard-bred New Hampshires.* Small numbers of Rhode Island Reds, Silver Cornish crosses, Single-Comb White Leghorns, Leghorn-Red crosses, and Buff Australorps were mixed with the New Hampshire chicks when

From the Animal Disease and Parasite Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md.

The *beta*-propiolactone used in this study was supplied by the B. F. Goodrich Chemical Co., a division of the B. F. Goodrich Co., Rose Building 3060 E. Ninth Street, Cleveland 15, Ohio.

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* Obtained from the U.S.D.A. Animal Husbandry Research Division.

they were placed under the electric brooders at 1 day of age. Although all chicks came from dams vaccinated with a commercially prepared, formalin-inactivated, aluminum hydroxide-adsorbed Newcastle disease vaccine, significant quantities of serum-neutralizing (SN) and hemagglutination-inhibition (HI) antibodies were not demonstrated when six pools of serums from 30, 1-day-old chicks were assayed.

Facilities.—Throughout the course of this study, the birds were housed in a 15- by 25-ft. frame building with a concrete floor. A litter of wood chips or sawdust was maintained at all times.

The experimental vaccines were produced and evaluated in the Newcastle disease building, a secure isolation unit, designed for work with virus diseases of poultry.

Strains of Newcastle Disease Virus.—The three strains of NDV used were the Manhattan-Kan-1948 strain,† the Roakin-NJ-1946 strain,† and the GB-Texas-1948 strain (maintained in this laboratory since November, 1951).

Only lots of virus that exhibited a mean death time conforming to that established for the virus strain at Wisconsin ultimately were used in vaccine production. As used in these vaccines, each strain of NDV represents the first embryo passage in this laboratory.

Newcastle Disease Vaccines.—A single lot of experimental vaccine was prepared from each of the NDV strains described, each vaccine being in essence an 80 per cent suspension of whole embryo and fluids, minus albumin, in a crystal violet solution (1 Gm. crystal violet in 400 ml. ethylene glycol).

Ten- or 11-day-old embryonating chicken eggs were inoculated with a 10^3 dilution of seed virus and placed in a 99-F. egg incubator. Twenty-four hours after inoculation, all eggs were candled and those containing dead embryos were discarded. The remaining eggs were candled at hourly intervals after 40 hours of incubation. The 10^3 dilution of the GB-Texas strain produced 100 per cent embryo mortality in less than 40 hours; all embryos died within 45 hours after inoculation with the Roakin-NJ strain and within 43 hours after inoculation with the Manhattan-Kan strain. Eggs containing dead embryos were removed from the incubator as they were observed at the time of the hourly candling and chilled in the refrigerator. Each batch was harvested separately, collected in individual containers, and ground in an Eppenbach mill, set at 76, for three minutes. The resulting tissue suspension was filtered through several layers of sterile gauze, and the filtrate was collected in 400-cc. amounts in 500-cc. bottles. Aliquots of this material were obtained for sterility tests and virus titrations. Crystal violet-ethylene glycol solution (100 cc.) was then added. The resulting material was treated with a sufficient amount of a 10 per cent solution of BPL to yield a final concentration of 0.1 per cent. Inactivation was accomplished at 70 F. in two hours.

† Obtained from the Newcastle Disease Virus Repository, University of Wisconsin, Madison.

Safety tests on the finished product included bird and embryo inoculations.

EXPERIMENTAL PROCEDURE

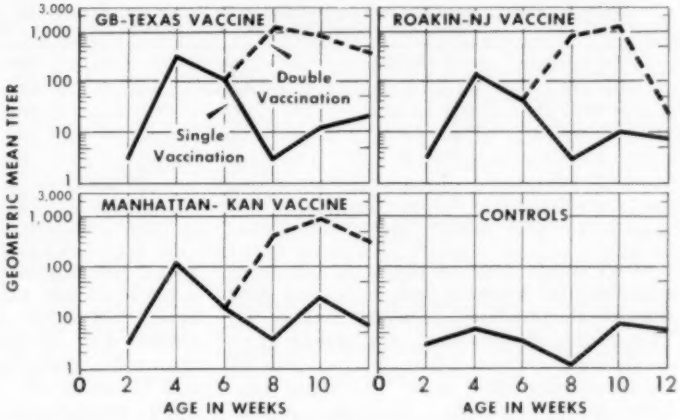
Vaccination Program.—At the age of 1 day, 1,100 chicks were allotted to four main groups and placed in separate houses. When they were 14 days old, all chicks except those maintained in the control group were vaccinated with one of the three experimental vaccines. Half of each group were revaccinated with the identical vaccine four weeks later, and the singly and doubly vaccinated birds were kept separate by a complete wire partition in each house. In all cases, 0.5 ml. of vaccine was injected into the subcutaneous tissue of the neck.

Vaccine Evaluation—Serological Tests.—Prevaccination blood samples were obtained from chicks when they were 1 and 14 days of age. Postvaccination blood samples were obtained at biweekly intervals from 5 birds selected at random from the singly and doubly vaccinated birds in each group. After sampling, the samples were allowed to clot, the serum was collected, filtered through Boerner centrifugal filters, and stored in the refrigerator until tested. (With the exception of the pooled sample obtained when the birds were 1 day old, all samples were collected, stored and tested separately.)

The *alpha* procedure was used in determining the HI titers.[22] The individual values obtained were converted to geometric mean titers (GMT) in accordance with the method of Markham.[19] Serum-neutralization titers obtained were treated in the same way in the final compilation. The GB-Texas strain of NDV was used in these tests.

Challenge Exposure.—A lot of 25 birds from each group of chickens which had received either single or double injections of one of three BPL-killed vaccines, as well as a similar lot from the control group, was brought to the Newcastle disease building at intervals of two weeks to be challenged with the GB-Texas strain of NDV. All birds were given 0.1 ml. of a 10^3 dilution of the challenge virus into the muscles of the thigh. At the same time, the $b.l.d._{50}$ (50 per cent bird lethal dose) of the challenge virus was established by injecting unvaccinated control chickens with virus dilutions of 10^6, 10^7, 10^8, and 10^9, using 3 chickens for each dilution, and the value computed according to the method of Reed and Muench. During the course of the experiment, the GB-Texas strain of NDV used in challenge and serological tests showed only a minor variation in $b.l.d._{50}$, $e.l.d._{50}$, and the hemagglutination (HA) titers.

Virus Isolation Attempts.—In order to obtain more knowledge on the status of the immunity in chickens vaccinated with BPL-killed Newcastle disease vaccines, attempts were made to recover the NDV from the blood streams of a representative number of birds after challenge The basic object of these trials was to determine the ability of the challenge virus to multiply in the blood of the vaccinated chickens. If the virus was not recovered from the blood and if the bird survived



Graph 1—The comparative hemagglutination-inhibition titers of several Newcastle disease vaccines. Single vaccinations were given when chickens were 2 weeks old, and double vaccinations were given at 2 and 6 weeks of age.

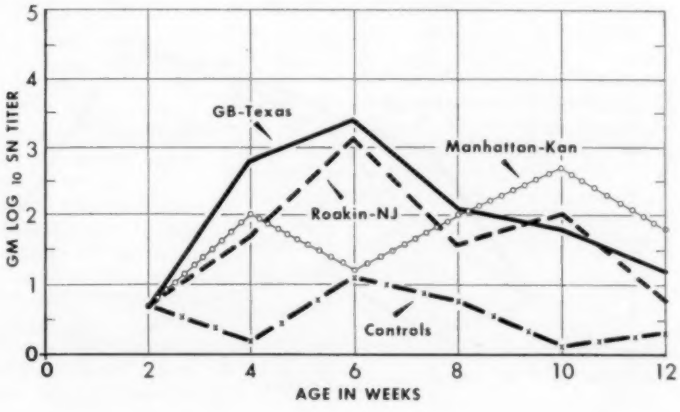
the challenge without signs of Newcastle disease, the chicken was considered resistant to Newcastle disease. The method of Hofstad[8] was used in all postchallenge virus recovery attempts, heparinized blood samples being taken 72 hours after challenge exposure. The washed erythrocytes were used to inoculate 10- or 11-day-old viable chicken embryos. Chorioallantoic fluids obtained from all embryos dying 24 hours or more after injection were tested for sterility and HA ability. Those showing a positive HA were considered infected with NDV.

RESULTS

The Newcastle disease HI titers obtained from the various groups of chickens before vaccination and revaccination and at later periods are shown (graph 1). Revaccination at 6 weeks of age resulted in an appreciable increase in HI levels, as compared with singly vaccinated groups of chickens. The antibody titers obtained in groups of birds that were given two injections of vaccine lasted for longer periods of time and reached their highest levels when the chicks were 10 weeks old.

The Newcastle disease SN titers obtained before vaccination and at biweekly intervals after single vaccinations are shown comparatively (graph 2). Results indicate that the experimental vaccine made from the GB-Texas strain produced the greatest serological response, and was followed in its effect by that made from the Roakin-NJ strain. Comparative SN titers of doubly vaccinated chickens are shown (graph 3). It is evident that the peak of virus-neutralizing ability, like the HI ability, was reached with all three vaccines at about 10 weeks of age and diminished thereafter.

Resistance to challenge, expressed in percentages of protection of chickens vac-



Graph 2—Comparative serum-neutralization titers of chickens given single vaccinations with killed Newcastle disease vaccines at 2 weeks of age.

TABLE 1—Resistance to Challenge of Chickens Vaccinated Singly and Doubly with BPL-Killed Newcastle Disease Vaccines, Expressed in Percentage Values

Age of birds (weeks)	GB-Texas		Roakin-NJ		Manhattan-Kan		
	Single vacc.	Double vacc.	Single vacc.	Double vacc.	Single vacc.	Double vacc.	Controls
4	100	.....	96	.....	96	.....	4
6	100	.....	92	.....	96	.....	8
8	80	100	96	100	88	100	0
10	100	100	76	95	92	100	16
12	85	100	90	100	65	100	15

cinated singly and doubly with the three experimental vaccines and compared with that of the controls, is shown (table 1). This study showed that, ten weeks after single vaccinations, 65 to 90 per cent of chickens vaccinated with the three BPL-killed vaccines resisted challenge. A substantially higher degree of protection (95 to 100%) was afforded by revaccination.

Results of attempts to isolate the virus from the blood of birds challenged with virus by the intramuscular route are shown (table 2). In most instances, the virus could not be recovered from the blood 72 hours after challenge of singly or doubly vaccinated chickens. Attention is directed to the large number of positive isolations obtained from the control group.

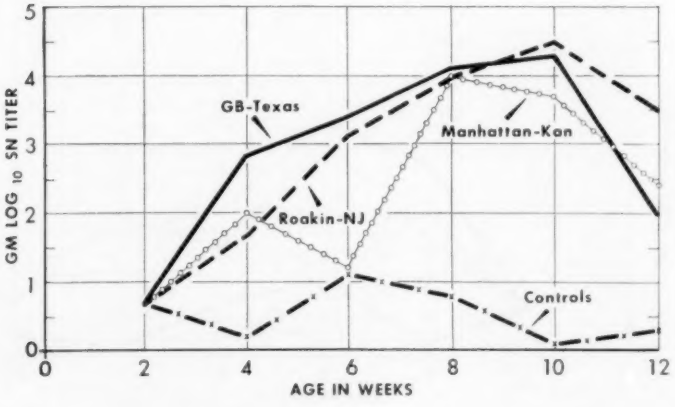
DISCUSSION

The experiments described in this paper demonstrate the degree and duration of immunity following the use of a single or double dose of three BPL-inactivated Newcastle disease vaccines made with the GB-Texas, Roakin-NJ, and Manhattan-Kan strains of NDV.

In general, groups of birds receiving only a single injection of these vaccines at 2 weeks of age exhibited a significant degree of immunity to Newcastle disease, as measured by the challenge exposure studies conducted when the birds were 4, 6, 8, 10, and 12 weeks old.

The vaccine prepared with the GB-Texas strain appeared to offer a slight advantage over vaccines prepared with either the Roakin-NJ or Manhattan-Kan strains of NDV, the former vaccine protecting 80 to 100 per cent of the birds challenged while the latter two vaccines protected 76 to 96 per cent and 65 to 96 per cent, respectively. When considering the slight advantage resulting from the use of the vaccine prepared with the GB-Texas strain of NDV, it should be recalled that a homologous challenge strain of NDV was used. The increased susceptibility of all groups of birds at the 12-week challenge may be considered an indication of the comparatively short duration of immunity afforded by a single injection of these vaccines.

Additional verification of these conclusions is furnished by the results of post-challenge virus recovery attempts and of serological tests that supplemented the challenge studies. Newcastle disease virus



Graph 3—Comparative serum-neutralization titers of chickens given double vaccinations with killed Newcastle disease vaccines at 2 and 6 weeks of age.

was recovered from the blood stream of 1 of 15 birds given the vaccine prepared with GB-Texas strain, from 2 of 16 birds given the vaccine prepared with the Manhattan-Kan strain, and from 2 of 16 birds given the vaccine prepared with the Roakin-NJ strain of NDV. The virus was recovered from 15 of the 16 unvaccinated birds.

Birds given a single injection of vaccine made from either the GB-Texas or Roakin-NJ strains of NDV exhibited significant HI and SN titers for a short time after vaccination; vaccine prepared with the Manhattan-Kan strain failed to elicit a significant serological response.

Birds given a second, or booster, injection of vaccine, showed increased resistance to challenge and a heightened serological response, irrespective of the viral strain incorporated in the vaccine. Similar observations regarding the anamnestic response to a second injection of inactivated Newcastle disease vaccine previously have been reported by Brandly *et al.*,[1] Waller and Gardiner[23] and, more recently, by Hofstad,[5,6] who emphasized the importance of the time interval between injections in establishing the optimum response. In the present study, a second, or booster, injection of vaccine administered four weeks after the initial injection produced an increased resistance to challenge that could be measured serologically. Only 1 of the 180 birds given a second injection of vaccine succumbed to challenge. This bird was given two injections of vaccine made with the Roakin-NJ strain of virus.

Additional evidence of a heightened state of immunity was indicated by our failure to recover the virus from any of the birds that were given two injections of vaccine. The failure to recover virus after challenge of vaccinated birds is in contrast to the results obtained by Hofstad,[7] who, working with formalin-inactivated vaccines, ultraviolet-irradiated vaccines, and a vaccine inactivated by prolonged incubation at 37 C., succeeded in isolating NDV from the blood of vaccinated birds in spite of high levels of antibody in the serums. The HI and SN antibodies persisted for longer periods of time in birds vaccinated twice with any of the three vaccines than in those singly vaccinated.

It has been observed, in experiments not included in this report, that BPL-inactivated Newcastle disease vaccine produced higher immune responses in chickens than similar products killed with formalin. The immunity could be maintained even when an excess of the minimal effective virucidal concentration was used.

TABLE 2—Results of Virus Isolation Attempts After Challenge of Singly and Doubly Vaccinated Chickens

Age of birds (weeks)	GB-Texas		Roakin-NJ		Manhattan-Kan		Controls
	Single vacc.	Double vacc.	Single vacc.	Double vacc.	Single vacc.	Double vacc.	
6	3/0*	../..	4/0	../..	4/0	../..	4/4
8	4/1	4/0	4/2	4/0	4/1	4/0	4/4
10	4/0	4/0	4/0	4/0	4/0	4/0	4/3
12	4/0	4/0	4/0	4/0	4/1	4/0	4/4

* Denominator = No. attempts made; numerator = No. birds positive.

SUMMARY

The results of studies of the comparative immunizing potency of three *beta*-propiolactone (BPL)-inactivated Newcastle disease vaccines, prepared with the GB-Texas, Roakin-NJ, and Manhattan-Kan strains of Newcastle disease virus (NDV), are presented.

A single injection of these vaccines afforded variable degrees of protection to groups of birds challenged at 4, 6, 8, 10, and 12 weeks of age. The vaccine made with GB-Texas strain of NVD afforded the best protection. Groups of birds given a second, or booster, injection of vaccine showed a characteristic anamnestic response that could be measured serologically and by postvaccination virus exposure. When two injections of vaccine were used, no significant difference could be observed in the comparative results of the serological and challenge tests of the various groups of birds, irrespective of the strain of virus used in the vaccine.

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Experimental Treatment of Infectious Sinusitis in Turkeys with Nitrofurans and Antibiotics

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NUMEROUS REPORTS on infectious sinusitis (IS) of turkeys can be found in the literature. This condition is generally considered to be caused by a pleuropneumonia-like organism (PPLO) and is closely related to chronic respiratory disease (CRD) of chickens.

In 1949, Hitchner [9] reviewed the literature concerned with the various treatments that had been used against this disease. There was apparent agreement that a 4 per cent aqueous solution of silver nitrate was the treatment of choice. Later reports by McNeil and Hinshaw,[11] and Bryan *et al.*[1] have given support to the value of the silver nitrate treatment. In most instances, there is considerable swelling, inflammation and, sometimes, necrosis of the sinus following injection of this material. Dickinson and Hinshaw,[2] however, reported that there was no permanent damage if care was exercised in the dosage and the injection site. Nevertheless, there has been a desire on the part of the pathologist and the poultryman to find a treatment just as effective and with less tissue damage.

Antibiotics were first used as treatments in 1949, when Hitchner [9] reported that the injection of 150 mg. of streptomycin in aqueous solution into the affected sinus caused complete recession of the swelling in seven to ten days in 20 of 21 turkeys, and that the other recovered with a second treatment. Draining the sinus prior to the injection of the drug did not appear to be of any benefit. McArther [10] likewise injected 100 to 150 mg. of streptomycin into the sinus and found that 90 per cent of 300 isolated birds recovered in five to seven days after injection. Most of the remaining birds recovered with a second treatment. Olson [12] found 100 mg. of streptomycin to be just as effective as 150 or 200 mg. when injected into the sinus without prior aspiration; dihydrostreptomycin appeared to be as effective as streptomycin. Grumbles and Boney [6] used streptomycin in amounts varying from 50 to 200 mg., and chlortetracycline (Aureomycin*) in amounts from 12.5 to 50.0 mg. injected into the infected sinus without prior aspiration. Van Roekel *et al.*[15] reported 50 to 150 mg. of streptomycin to be the most effective treatment for local sinus infection due to the CRD agent, and 25 to 50 mg. of chlortetracycline to be effective in some cases. Prier [13] observed that the injection of 50 mg. of chlortetracycline into the affected sinus eliminated the swelling in 8 of 13 naturally infected birds, but that 100 mg. injected into the muscle failed to alleviate signs in naturally or experimentally infected birds. Gross [5] determined that oxytetracycline (Terramycin†) in mineral oil was superior to either streptomycin or oxytetracycline in water when injected into the sinus.

Grumbles and Boney [7] reported that levels of 0.50 and 0.25 per cent of chloramphenicol (Chloromycetin §) in an all-mash diet for eight to 12 days produced apparent recovery in all birds by the seventh to eleventh day after treatment, while a 0.5 per cent level of oxytetracycline in an all-mash diet was not as effective. Francis [4] observed that chlortetracycline fed at the rate of 100 Gms. per ton of feed prevented the development of the disease in the half of a turkey flock of 600 receiving it, while the half not receiving the medicament developed the disease.

Domermuth and Johnson [3] tested 12 drugs for their *in vitro* activity against an avian strain of PPLO and found that furazolidone (Furoxone**) ranked with oxytetracycline and streptomycin in ability to kill PPLO, although all three ranked below carbomycin (Magnamycin‡). Schmittle and Putnam [14] have recently reported on the efficacy of furazolidone in the treatment of IS and found it to be superior to streptomycin.

The experiments reported in this paper were undertaken in an effort to determine the relative efficacies of several of the nitrofurans, two of the antibiotics, and silver nitrate in the treatment of IS.

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* Aureomycin is produced by American Cyanamid Co., New York, N. Y.

† Terramycin is produced by Pfizer Laboratories, Brooklyn, N. Y.

§ Chloromycetin is produced by Parke, Davis and Co., Detroit, Mich.

** Furoxone is produced by Eaton Laboratories, Norwich, N. Y.

‡ Magnamycin is produced by Pfizer Laboratories, Brooklyn, N. Y.

MATERIALS AND METHODS

In each experiment reported, Beltsville White turkeys were obtained as day-old poults. These poults were raised in electric brooders to sufficient age for experimental purposes. All turkeys were vaccinated for Newcastle disease at 4 weeks of age with 0.5 ml. of a killed vaccine (Wyeth) injected into the thigh muscle. Just prior to the introduction of the infectious material, the turkeys were distributed at random in all-metal 6 by 6 ft. range shelters called "rangers." The design of each experiment was such as to include a control group of uninoculated, untreated turkeys, which were removed from the general infected area, and a control group of inoculated, untreated turkeys.

The disease was produced in experiment 1 by injecting the left sinus with 0.5 ml. of a broth suspension of air sac and exudate obtained from a clinical case of CRD of chickens. This inoculum had been prepared by grinding the material with sterile nutrient broth in a mortar and pestle. It was held at —15 C. until inoculated. This injection was preceded by the administration of yolk material from egg passage of PPLO in which 0.25 ml. was given intratracheally and 0.25 ml. into the sinus one week earlier. No signs were observed in any of the challenged birds prior to the administration of the air sac inoculum. In experiment 2, the disease was produced by injecting the left sinus with 0.5 ml. of a broth suspension of material previously aspirated from the untreated sinuses of turkeys suffering from the condition. This material was prepared by diluting the sinus exudate approximately 2:1 with sterile nutrient broth. It was held at —15 C. until injection. All turkeys were observed daily from the time of inoculation with the infectious material until the termination of the experiment.

In both experiments, visible signs of swollen sinuses, watery eyes, and coughing were in evidence within six to seven days after injection of the infective material. Such signs increased in severity until a majority of the inoculated turkeys were involved. Treatment was begun at the time that it was felt that treatment normally would have been started under field conditions and when it was deemed that sufficient development of the disease had occurred to permit evaluation.

At the time of treatment, each turkey (with the exception of those of the untreated, uninoculated group) was handled individually and rated as to the degree of swelling on the basis of gross sinus involvement. The uninoculated control group was also observed for signs of infection at this time. The ratings were based, in general, on the method described by Olson.[12] In our experiments, a system of ratings of "none," "light," "moderate," and "severe," corresponding to 0, 1, 2, and 3, respectively, was used in evaluating the sinus involvement. A rating of "light" indicated a slight swelling containing a small amount of fluid, while "severe" was a swelling usually sufficient to close the eyelids, and "moderate" was a swelling between these two. The observations and ratings in all groups were made by the same individual, who did not have prior knowledge of the treatment to be used. Such data were then used to obtain a geometric rate (g.r.) of sinus swelling prior to treatment. All involved sinuses were aspirated after examination and before treatment in all groups, including the untreated, inoculated control group. Such a procedure was undertaken in order to make all groups as nearly equal as possible at the beginning of the observation period.

In the administration of the dust compounds used in experiment 1, the material was placed in a Hudson duster and dusted among the turkeys in the rangers. No means were taken to confine the material, as this was not considered typical of the manner in which the dust would be handled under field conditions. The administration of the soluble drug was by the usual means, with medicated water being the only source of water for the duration of the treatment period. A fresh solution of the soluble medicament was prepared daily. Drugs were injected into the sinuses with an 18-gauge hypodermic needle attached to a 10-cc. syringe. Both sinuses were injected in each turkey of the groups which were treated in this manner.

An observation period of 21 days followed the initial treatment. During this time all turkeys were observed daily. All deaths were recorded and all dead turkeys were necropsied to determine the cause of death.

At the end of the 21-day observation period, all turkeys were killed and necropsied. At this time, the involvement of the sinus was again recorded on the basis of the external swelling and upon the amount of mucus and inflammatory change present within the sinus. The same system of rating was used as when the sinuses were observed prior to treatment. The pathological changes present in the air sacs were also recorded with a designation of 1+ to 4+ according to the degree of change observed. The 1+ indicated only slight clouding and thickening of the air sacs, while a 4+ designated considerable thickening and the presence of exudate in the majority of the air sacs; 2+ and 3+ were gradations between 1+ and 4+. Any mortality occurring during the observation period and determined to be due to the disease was counted as the highest possible degree of pathological change in the final analysis. Such data were then used to determine the g.r. of involvement, which is used to express the results in these experiments. This figure is actually a percentage factor obtained by dividing the total actual involvement by the total possible involvement, *i.e.*, 20 birds with a possible involvement of 3 per bird (as in the sinusitis rating) would have a total possible involvement of 60. Assuming that the actual involvement was 2 per bird, this would be a total actual involvement of 40. The g.r. of this group on a pen basis would then be 40 divided by 60 (66.66%).

TABLE 1—Degree of Pathological Change After 21 Days' Treatment for Infectious Sinusitis (Trial 1)

Ranger* (No.)	Treatment	g.r.† of sinusitis before treatment	g.r. of sinusitis at termination	Change in g.r. of sinusitis	g.r. of aerosacculitis
1	No treatment, inoculated controls	22.8	74.0	+52.0	45.0
2	Furazolidone aerosol	40.3	61.0	+21.0	34.0
3	Nitrofurazone-furazolidone water mix	28.0	63.0	+35.0	47.0
4	Furazolidone in mineral oil	40.3	33.0	− 7.0	15.0
5	Furazolidone suspension	52.6	26.0	−26.0	29.0
6	Streptomycin-dihydrostreptomycin combination	42.1	29.0	−13.0	20.0
7	No treatment, uninoculated controls	00.0	00.0	.....	00.0

* 18 turkeys in each ranger; † geometric rate.

Experimental Procedure

In experiment 1, 126 turkeys were distributed at random to seven rangers, with 18 birds being placed in each ranger. The infective material was inoculated when the turkeys were 14 weeks old. Signs of infectious sinusitis were observed within seven days of inoculation, and treatment was begun 11 days after the injection of the infectious inoculum and four days after the first signs were observed.

The drugs used in this experiment were furazolidone (25% micronized) as an aerosol, 2 Gm. daily for five days; 4.5 per cent nitrofurazone (Furacin*) and 1.77 per cent furazolidone as a water mix, 16 Gm. in 2.5 gal. of water for the entire 21-day observation period; and 10 per cent furazolidone in mineral oil, in suspension, and a streptomycin-dihydrostreptomycin combination (Bi-strep†) injected in a 0.5-ml. quantity into each sinus.

In experiment 2, using the same design as in the first experiment, 180 turkeys were distributed at random to nine rangers, with 20 birds being placed in each ranger. The infective material was inoculated when the turkeys were 7 weeks old, and signs of IS were apparent within six days and increased in severity until treatment started. Treatment was initiated 14 days after inoculation and eight days after signs were first noticed (table 2). Drugs used in this experiment were: 10 per cent furazolidone in suspension and in mineral oil, 10 per cent nitrofurantoin (Furadantin§) in water miscible base and in mineral oil, oxytetracycline in mineral oil, streptomycin and dihydrostreptomycin combination, and silver nitrate (4% in aqueous solution). The furazolidone and nitrofurantoin suspensions in mineral oil and the silver nitrate solution were prepared just prior to use. All drugs were injected into the sinuses, with 1-ml. quantities of each preparation being injected into each sinus. Within ten minutes after the injection of the silver nitrate solution, a severe swelling, which was sufficient to close the eyelids, was observed. Rangers 2 and 7 had only 19 turkeys at the time of treatment.

* Furacin is produced by Eaton Laboratories, Norwich, N. Y.

† Bi-strep contains 125 mg. streptomycin and 125 mg. dihydrostreptomycin per cubic centimeter, and is produced by Delaware Poultry Laboratories, Millsboro.

§ Furadantin is produced by Eaton Laboratories, Norwich, N. Y.

Results

The degree of sinus involvement prior to treatment, at termination, and the increase or decrease in sinus involvement, as well as the degree of aerosacculitis, are given for each experiment (tables 1, 2).

In experiment 1, the greatest increase in the g.r. of sinusitis occurred in the untreated, inoculated control group. Those groups treated with furazolidone as an aerosol and the nitrofurazone-furazolidone water mix also showed an increase in the g.r. of the sinusitis and the greatest amount of aerosacculitis. In general, there was a decrease in the g.r. of sinusitis in the groups in which drugs were injected into the infra-orbital sinus (furazolidone in suspension and in mineral oil, and streptomycin-dihydrostreptomycin combination) and these groups also showed the least amount of aerosacculitis. One turkey from ranger 1 died 15 days after injection of the infective inoculum and four days after treatment. Death was determined to be due to IS. No signs of sinusitis were seen in the right sinus at necropsy. The uninoculated control group was completely free of any degree of sinusitis or aerosacculitis.

In experiment 2, an increase in g.r. of sinusitis was seen in the untreated, inoculated control group, the nitrofurantoin-treated group, the streptomycin-dihydrostreptomycin-treated group and the silver nitrate-treated group, while all other

TABLE 2—Degree of Pathological Change after 21 Days' Treatment for Infectious Sinusitis (Trial 2)

Ranger* (No.)	Treatment	g.r.† of sinusitis before treatment	g.r. of sinusitis at termination	Change in g.r. of sinusitis	g.r. of aerosacculitis
1	No treatment, inoculated controls	38.0	67.0	+29.0	36.00
2	Furazolidone suspension	42.0	33.0	— 9.0	12.00
3	Furazolidone in mineral oil	47.0	38.0	— 9.0	36.00
4	Nitrofurantoin suspension	35.0	45.0	+10.0	40.00
5	Nitrofurantoin in mineral oil	38.0	23.0	—15.0	26.00
6	Oxytetracycline in mineral oil	40.0	37.0	— 3.0	39.00
7	Streptomycin-dihydrostreptomycin combination	33.0	63.0	+30.0	36.00
8	4% silver nitrate in aqueous solution	37.0	50.0	+13.0	50.00
9	Uninoculated control, no treatment	00.0	00.0	.....	1.25

* 20 turkeys in all rangers except 2 and 7, which had only 19 turkeys each; † geometric rate.

groups showed a decrease of sinusitis. Except for a few birds, the condition was confined to the inoculated sinus. In the control of the aerosacculitis and nitrofurantoin in mineral oil showed the least amount of pathological change.

DISCUSSION

It is apparent that some differences exist in the results of the two experiments. The fact that the disease was induced by different inocula and under somewhat different weather conditions may account for some degree of the differences.

Those drugs which were administered by dusting or in the water had little or no effect on the course of the disease. The results obtained when mineral oil was used as a vehicle would seem to lend support to earlier observations that suspension of the drug adds to its effectiveness. As the necrosis produced by the silver nitrate solution was most severe in the sinus which was not involved, it would appear that the exudate in an affected sinus serves to dilute or otherwise curtail the caustic action of this drug.

Finally, the apparent ineffectiveness of streptomycin-dihydrostreptomycin combination in the second experiment was in sharp contrast to that obtained in the first experiment and with the observations of most workers who have used streptomycin. The drug used in both experiments was from the same container; the only difference was the length of storage time. The drug was stored, without refrigeration, in the same manner as the other drugs used in the experiments.

SUMMARY

Two consecutive trials on the experimental treatment of infectious sinusitis (IS) of turkeys are reported. Several of the nitrofurans administered by dusting, in the drinking water, and injection into the sinuses, two antibiotics injected into the sinuses, and 4 per cent aqueous solution of silver nitrate injected into the sinuses were used in these trials. Those drugs which were injected directly into the sinus were more effective (with the exception of silver nitrate and streptomycin-dihydrostreptomycin combination in experiment 2) than drugs administered by dusting or in water.

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The Effects of Continuous Sulfaquinoxaline Feeding on Rabbit Mortality

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BEFORE DOMESTIC rabbits were raised in sufficient numbers to be of commercial importance, mortality did not attract much attention, but as soon as attempts were made to produce rabbit meat in larger volume, it became an important factor. It was estimated that more than $900,000 was lost each year by commercial rabbit growers in the Los Angeles area alone because of mortality just before marketing age.[9]

Cost-accounting studies on rabbit production have been conducted in Los Angeles County, California, by the Agricultural Extension Service of the University of California at Berkeley since 1930. During the ten-year period of 1930 to 1939, those breeders cooperating sustained an average loss of 23 per cent of the unweaned young and 19 per cent of the mature stock.[10] The reports gave no details as to the causes of death.

Since World War II, several additional counties in California have made similar studies. In the southern California area, the cost studies for the years 1947 to 1949, inclusive, showed an average mortality of 22 per cent of the unweaned young. The average mortality for all mature stock was not included, but for does, alone, the figure reported was 35 per cent.[7,8]

Lund,[5] in his mortality study at the U. S. Rabbit Experiment Station, has provided a tentative standard for comparing losses. During the years 1947 to 1949, inclusive, mortality statistics were kept on 2,083 litters, from which 13,915 young rabbits were retained. Of these young, 19.6 per cent were lost by the end of 56 days, the age at which they are usually marketed as fryers. The average mortality for mature stock was 24.8 per cent. Statistics show that three causes of mortality are significant. These are enteritis, pneumonia, and miscellaneous (occurring in the nest boxes). During this three-year period, of the 2,726 (19.6%) young lost, almost 50 per cent had enteritis in some form. This represents approximately a 10 per cent average loss from enteritis among the retained young. Pneumonia was present at death in 18 per cent of the animals under 56 days of age and in 53 per cent of the mature animals. During the first three weeks of life, mortality was 8.3 per cent in nest boxes.

TABLE 1—The Effect of Feeding Sulfaquinoxaline on the Number of Deaths Due to Enteritis

Types of enteritis	Sulfaquinoxaline lots		Control lot		Expected *	
	Distribution (%)	Av. age at death (days)	Distribution (%)	Av. age at death (days)	Distribution (%)	Av. age at death (days)
Diarrheal	91.7	46	91.0	45	82.5	44
Mucoid	5.6	43	0.0	0	11.1	45
Hemorrhagic	2.7	37	9.0	40	6.4	40
	100.0	42 Av.	100.0	43 Av.	100.0	43 Av.

* From statistical data by Lund.[5]

METHODS

Since sulfaquinoxaline was found to be an efficient drug for the control of the liver type of coccidiosis in the rabbit[1,4,6] and to be effective, as reported by Delaplane[2] and Eveleth *et al.*,[3] in decreasing the numbers of deaths due to *Pasteurella multocida* infection in poultry, it was decided to study the effect of continuous sulfaquinoxaline feeding on rabbit mortality.

Twenty white New Zealand does were housed in individual all-metal hutches. The ration was hopper-fed, and water was furnished by a "dew-drop" system. Sulfaquinoxaline (0.5 lb.) was incorporated into a standard 18 per cent protein rabbit pellet to make a final percentage of 0.025

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The sulfaquinoxaline used in these experiments was furnished by Dr. Laurent Michaud, Merck & Co., Inc., Rahway, N. J.

TABLE 2—The Effect of Feeding Sulfaquinoxaline on the Number of Deaths Due to Pneumonia

	No. of animals	No. of deaths from all causes	Death from primary pneumonia			Death from enteritis and pneumonia		
			(No.)	(%)	Percentage of total mortality	(No.)	(%)	Percentage of total mortality
Sulfaquinoxaline-fed lots	367	100	0	0.0	0.0	8	2.2	7.4
Control lot	362	87	11	3.0	12.6	7	1.9	8.0
Expected *	13,915	2,726	147	1.0	5.3	293	2.1	10.7

* From statistical data by Lund.[5]

sulfaquinoxaline. Sulfaquinoxaline feeding was started in five lots, so that the young would be introduced to the ration at ages 0, 7, 14, 21, and 28 days.

A control lot of 20 does was set up at the same time and housed in a similar manner. The control ration was the same as that for the sulfaquinoxaline-fed lots except it contained no sulfaquinoxaline.

Deaths were recorded, and organs were cultured for Pasteurella in all lots. The young were weaned at 56 days of age and were removed from the experiment at that time.

RESULTS

The does fed sulfaquinoxaline produced 50 litters, from which 367 young were retained and 267 (73%) were weaned. The does in the control lot produced 45 litters, from which 362 young were retained and 275 (76%) were weaned. In the sulfaquinoxaline-fed lot, the weaned young averaged 3.84 lb., whereas in the control lot, the average weight when weaned was 3.75 lb.

The addition of sulfaquinoxaline to the feed had no effect on reducing the mortality due to enteritis. In the sulfaquinoxaline-fed lot, 15 per cent had enteritis, which accounted for 52 per cent of the total mortality; whereas in the control lot, 12 per cent had enteritis, which accounted for 53 per cent of the total mortality. Distribution of the types of enteritis and the average age of death are shown (table 1).

The incidence of the hemorrhagic type of enteritis was lowered by the sulfaquinoxaline feed.

In the control lot, there were 11 cases of primary pneumonia and seven cases of pneumonia secondary to enteritis. There were no cases of primary pneumonia in the sulfaquinoxaline-fed lots, but there were eight cases of secondary pneumonia. Although the sulfaquinoxaline had eliminated the primary pneumonias in this test, it had no effect on the enteritis-pneumonia complex (table 2).

Pasteurella multocida was isolated from 50 per cent of all rabbits with pneumonia in the control lot. Although the number of enteritis-pneumonia cases was not reduced by feeding sulfaquinoxaline, the number of Pasteurella isolations was reduced by 70 per cent. The effect of continuous sulfaquinoxaline feeding can be measured by fryer mortality studies and bacteriological cultures, but its effect on pneumonia, chronic sinusitis, and reproductive infections in the mature animal can only be estimated.

Nest-box mortality from birth to 21 days of age is usually high. Many of the young are not strong enough to nurse under competitive conditions and hence die from starvation. Some starve because the doe's milk supply is inadequate or because the doe is reluctant to remain in the nest box

TABLE 3—Comparison for Nest-Box Deaths of Rabbits During the First Four Weeks of Life

	Lots fed sulfaquinoxaline beginning at—						
	21 to 28 days of age	14 days of age	7 days of age	Birth	Sulfaquinoxaline lot	Control lot	Expected *
Total No. of young	80	80	51	156	367	362	13,915
No. of nest-box deaths	6	4	4	18	32	30	1,160
Percentage of nest-box deaths	7.5	5.0	7.8	11.5	8.7	8.3	8.3

* From statistical data by Lund.[5]

long enough for the young to nurse properly. Trampling by the doe, exposure due to being caught in the wire flooring, and infections of the navel are also causes of nest-box deaths.

The age at which the young are introduced to the sulfaquinoxaline feed determines the effect of the drug on the nest-box mortality. When the young and their dams were fed the ration containing sulfaquinoxaline beginning at parturition, the mortality was greater than in the control lot. If the drug was introduced when the young were 7 to 14 days of age, then the mortality rate was less than expected and less than the rate of the control lot (table 3).

SUMMARY

1) A ration containing 0.025 per cent of sulfaquinoxaline was continuously fed to 20 white New Zealand does and 367 young over a 12-month period. No toxic effects from the drug were noticed in any of the animals under observation. At this level, the ration was palatable as indicated by the average weights of the weaned young.

2) The sulfaquinoxaline ration had no effect on the mortality caused by enteritis, but it did alter the type distribution. The hemorrhagic type of enteritis in the sulfaquinoxaline-fed lots was 70 per cent less than that in the control lot.

3) In this test, the sulfaquinoxaline ration eliminated deaths caused by primary pneumonia and reduced the number of *Pasteurella multocida* isolations by 70 per cent. The enteritis-pneumonia complex was not altered, but the number of Pasteurella isolated was significantly reduced.

4) Nest-box mortality was reduced when the drug was introduced to the doe and her young when the litter was 14 days of age. The average nest-box mortality remained the same in the control and sulfaquinoxaline-fed lots.

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The Growth and Cytopathogenicity of Vesicular Exanthema Virus in Swine Kidney Tissue Culture

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STUDIES WITH the vesicular exanthema virus (VEV) have been retarded, chiefly because of the inability to propagate the virus, except in swine, the natural host, and, irregularly, in dogs (Bankowski and Wood [3]). The difficulties and expense involved in the use of swine are great; thus, there has been a continuing search for a means of virus propagation and assay.

McClain *et al.*[5] reported the successful cultivation of VEV type B_{51} on fragment implants of swine embryo skin, muscle, tongue, and snout tissue, and postulated that swine kidney epithelial cultures would propagate this virus. Bankowski and Pfeifer [4] propagated VEV in a minced swine embryo suspension. Stuart and Bankowski [8] propagated the virus on xenoplastic grafts of swine embryo skin on the chorioallantoic membranes of embryonating chicken eggs. Sellers [7] reported the growth of two strains of VEV and the formation of plaques in pig kidney tissue culture. Sellers' paper deals primarily with foot-and-mouth disease virus and vesicular stomatitis virus, and he does not give any protocols or data regarding his VEV work.

The study reported herein was undertaken to find a tissue which would be more readily available than embryo tissue, and a tissue culture system which would be less tedious than the fragment implant culture system and which would lend itself to large-scale diagnostic use with the criteria of a cytopathogenic effect.

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MATERIALS AND METHODS

Tissue Culture Preparation.—Young swine from 1 to 16 weeks of age were killed, and a kidney from each was removed aseptically. When kidneys from older swine were used, the outgrowth of cells either was scanty or the cells failed to grow.

The capsule was removed from the kidney, and the cortex minced with scalpels. Cell clusters were produced by trypsinizing the fragments as described by Bachrach *et al.*[1] The final washed pellet of cell clusters was resuspended (1:200) in a nutrient fluid consisting of 5.0 per cent horse serum, 0.5 per cent lactalbumin hydrolysate, and 94.5 per cent Hanks' solution containing penicillin (100 units/ml.) and streptomycin (100 μg/ml.). This cell suspension was dispensed in 0.9-ml. amounts in 13- by 100-mm. tubes. The stoppered tubes in racks were incubated at 37 C. and set at an angle so that the fluid extended about one half way up the tube. After 48 to 72 hours, the nutrient fluid was aspirated and replaced with a nutrient fluid consisting of 94.5 per cent Earle's solution, 5.0 per cent horse serum, 0.5 per cent lactalbumin hydrolysate, and antibiotics at the same level as stated.

Virus inoculations were made when the cultures were 6 to 10 days old. The twentieth passage was made in Roux flasks which were seeded with 70 ml. of cell suspension and otherwise managed in the same manner as the tubes.

Virus.—The VEV type B_{51}, used for this experiment, was received in 1954 from a New Jersey outbreak, was passaged through swine five times, and was frozen in December of that year. The virus was typed by cross-immunity studies by this division. One additional passage in swine was made prior to the inoculation in tissue culture. The vesicular coverings from the passage pigs were harvested and ground by mortar and pestle

TABLE 1—Growth of Vesicular Exanthema Virus Type B_{51} in Tissue Culture

Passage No.	Total dilution	Tissue culture i.d.$_{50}$/0.1 ml. (neg. log)	Swine i.d.$_{50}$/1.0 ml. (neg. log)	Swine inoculation results	Swine challenge results
1	7	6.5	Not run	Not run	Not run
2	14	6.5	Not run	Not run	Not run
3	15	Not run	Not run	2/2*	0/2*
4	16	Not run	Not run	2/2	0/2
7	23	7.2	Not run	Not run	Not run
7	23	7.5	Not run	Not run	Not run
20	38	7.2	Not run	Not run	Not run
20	38	6.7	Not run	Not run	Not run
20	38	6.6	4.0	4/4	0/4
20	38	6.6	3.8	4/4	Not run

* The numerator equals the number of animals positive; the denominator, the number of animals inoculated.

TABLE 2—Serum Neutralization of Vesicular Exanthema Virus with Hyperimmune Serums in Tissue Culture

Swine (No.)	Dilution (serum)	Type (serum)	i.d.$_{50}$ virus titer (neg. log)	i.d.$_{50}$ of serum-virus mixture (neg. log)
247	1/5	B	6.75	0.5
247	1/10	B	6.75	0.5
247	1/20	B	6.7	1.5
2834	1/5	A	6.7	7.2
2834	1/10	A	6.7	6.5
2842	1/5	A	6.7	5.75
2842	1/10	A	6.7	5.75
3041	1/5	C	6.7	5.75
3041	1/10	C	6.7	6.5
3046	1/5	C	6.7	5.75
3046	1/10	C	6.7	5.75
3878	1/5	Normal	6.7	6.7
3878	1/10	Normal	6.7	6.7

to a concentration of 20 per cent in 2 per cent tryptose broth. Streptomycin and penicillin were added in concentrations of 5 mg. per milliliter and 5,000 units per milliliter, respectively. The virus was centrifuged at 3,000 r.p.m. for 20 minutes.

Passages were carried out both by titration passage and by serial passage. In each case the inoculum was 0.1 ml., so that the dilution factor in each serial passage was tenfold. In the titration passages, it was equal to the end point dilution times ten.

Neutralization.—Virus suspensions, harvested from Roux flasks, were diluted 1:5. Tenfold serial dilutions were made, and 0.5 ml. of the virus was mixed with 0.5 ml. of 1:5, 1:10, or 1:20 swine serum, which had previously been filtered through a Seitz microfilter and inactivated at 56 C. for 30 minutes. The swine serum and virus were diluted in 2 per cent tryptose broth. The mixture was held at room temperature for one hour, and 0.1 ml. was inoculated into each of three tissue cultures. The tissue cultures were then incubated at 37 C. and examined microscopically for cytopathogenic effects every 24 hours, up to 72 hours after inoculation, when the final reading was made. Sterility was checked by inoculation into Bacto-AC broth and 2 per cent serum agar. Calculations of 50 per cent end points were made according to the method described by Reed and Muench.[6]

TABLE 3—Serum Neutralization of Vesicular Exanthema Virus with Convalescent Serums in Tissue Culture

Swine (No.)	Days post-inoculation	i.d.$_{50}$ virus titer (neg. log)	i.d.$_{50}$ of serum-virus mixture (neg. log)
3562	0	7.5	6.5
3562	4	7.5	3.0
3562	10	7.5	0.5
3562	18	7.5	0.5
3565	0	7.5	6.5
3565	4	7.5	3.5
3565	10	7.5	0.5
3565	18	7.5	0.5

RESULTS

Vesicular exanthema virus type B_{51} was passaged 20 times on monolayers of swine kidney tissue culture cells (table 1). The first two passages and the seventh passage utilized the titration-passage technique, and the other passages were carried out serially. The total dilution of original infectious swine material after 20 passages was 10^{-38}. Passages 3 and 4, representing an effective dilution of original swine material of 10^{-15} and 10^{-16}, respectively, were inoculated into swine. Inoculations were made intradermally on the snout and all four feet or on the right front foot only, using 1.0 ml. of inoculum per site. All swine showed typical lesions of vesicular exanthema (VE) at each site within 48 hours. These swine were challenged after two months with known VEV and were not susceptible, while control swine developed VE.

The twentieth passage was also checked for virulence in swine and was titrated in swine and tissue culture concurrently. After 20 passages, the virus still produced typical lesions of VE in swine. The titers of this material in two trials in swine were $10^{-3.8}$ and 10^{-4} swine i.d.$_{50}$ per 1.0 ml., respectively. The titrations in tissue cultures of twentieth passage material ranged from $10^{-6.6}$ to $10^{-7.2}$ tissue culture i.d.$_{50}$ per 0.1 ml. This difference of 3.0 to 3.5 logs, based on doses as they were used, was probably due to the natural resistance of the swine.

The tissue cultures inoculated with VEV B_{51} showed a marked cytopathogenic effect in from 24 to 48 hours. This effect has been described by McClain *et al.*[5] for VEV, by Bachrach *et al.*[1] for vesicular stomatitis virus, and by Bachrach *et al.*[2] for foot-and-mouth disease virus. The cytopathogenic change is similar for each of these diseases and is characterized by the appearance of round cells, a shrinking of the cell, granulation, and pyknosis. The cells finally separate from the glass wall of the tube.

Typical results of the neutralization tests (table 2) show that the agent responsible for the cytopathogenic effect was neutralized by serums from swine which are immune to type B_{51}. This was confirmed 14 times with homologous serums, the minimum number of doses neutralized being 5.2 logs, representing a neutralization index of 160,000. The agent was not neutral-

ized, however, by serums from swine which were hyperimmune to type A_{48} or type C_{52}, as shown in four instances each. It also was not neutralized by normal swine serums or by serum from a horse which had been immunized with type A_{48}. The maximum number of doses neutralized by heterologous or normal serums was 0.95 log, which represents a neutralization index of less than ten. Neutralization results (table 3), with blood samples which were taken serially from 2 swine (No. 3562 and 3565), show that neutralizing antibodies are present in marked amounts within four days after inoculation of susceptible swine.

SUMMARY AND CONCLUSIONS

Swine kidney cells in tissue culture are capable of supporting the growth of vesicular exanthema virus (VEV) type B_{51}. This is demonstrated by the fact that, through 20 passages in such a system, representing a dilution from the initial material of 10^{-38}, the virus still manifests typical reactions in swine and in the neutralization test. In addition, the virus has a cytopathogenic effect on swine kidney cells in tissue culture which leads to their eventual destruction. This effect is readily observable by microscopic examination of cells. This cytopathogenic effect was used as the indicator of virus growth in an *in vitro* neutralization test. An early appearance of neutralizing antibody was demonstrated which would be of significance in the diagnosis of vesicular exanthema (VE). Although experimental work still is required to verify an assumption that the other types of VEV can be propagated in tissue culture, the use of such a tissue culture system would have great application in the diagnosis of VE, and in other laboratory investigations of the virus.

ADDENDUM

Since the completion of this report, similar work with vesicular exanthema virus (VEV) types A_{48} and C_{52} has resulted in the successful propagation of these types in tissue culture.

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Studies on the Agglutinative Substances in Anti-Brucella Serums

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IT IS KNOWN that marked changes in protein components of human and animal serums occur following certain bacterial infections. This is indicated by an increase in the amount of globulin, accompanied by a relative decrease in the albumin component in circulating blood.[7]

Weimer *et al.*[16] reported a similar phenomenon in Brucella antiserum, based on chemical analysis of the serum protein components.

It is also generally recognized that the anti-Brucella agglutinins move, electrophoretically, with globulins. Hess and Roepke,[8] using paper chromatography, demonstrated the presence of a nonspecific agglutinative substance in bovine serums obtained from brucellosis-suspicious herds.

This investigation was undertaken to determine further the character of these substances. The correlation between rising titers and changes in serum components was studied, using the ultracentrifuge and electrophoresis methods.

An attempt was also made to determine the nature of the agglutinative substance by applying a new method; the agglutination test on filter paper, where the serum components were separated by paper electrophoresis.

MATERIALS AND METHODS

Preparation of Antiserum.—Serums from 2 healthy 6-month-old rabbits, which did not show any agglutination titer against Brucella, were pooled and designated as serum A. Each rabbit was inoculated with 0.7 ml. of a saline suspension containing 3 mg. of wet viable Brucella strain K 18. Each animal was given 1.4 ml. of the same concentration of antigen on the third and sixth days after the initial injection. On the eighth day, 5 ml. of blood was drawn from each rabbit. This was repeated on the ninth day, and the serums obtained on both days were pooled and designated as serum B. Both animals were injected again on the twelfth day with 3 ml. of the original concentration of viable cells. Blood was again taken from each rabbit on the eighteenth day. These serums were designated as serum C. The agglutination titers of serums B and C were 1:3,200 and 1:6,400, respectively, using the antigen prepared in the following methods.

Brucella Tube-Agglutination Antigen (Strain 125).—Cultures were grown for 72 hours on agar medium with 10 per cent glycerin added. The growth was harvested in physiological saline solution and passed through cotton. The suspension was heated at 60 C. for one hour, diluted to a concentration of McFarland No. 1, and phenol was added to a final concentration of 0.5 per cent.

Bovine Serums.—Most of the samples were obtained from animals which had been detained in quarantine as brucellosis-suspicious, and which were examined every month by the tube-agglutination test. Both the International Standard Method * and the Japanese Standard Method † were applied. The titers of serums from 15 cattle which were used in the present experiment were approximately 1:80 ± by the International Standard Method and 1:200 ± by Japanese Standard Method. The other five serums were obtained from brucellosis-suspicious cattle which were killed at the institute. Their titers were as high as the other bovine serums.

Fractionation.—The methods for the fractionation of serum by differential precipitation with ammonium sulfate presented by Cohn *et al.*[4] were applied. Each sediment was dialyzed against 0.14 M sodium chloride saline solution for 24 hours at 4 C. They were then diluted with the same solution to original volume of serum.

Ultracentrifuge.—The ultracentrifugal investigation of rabbit and bovine serums was made in a Spinco model E ultracentrifuge. Each serum was diluted to a 1:5 dilution with 0.14 M sodium chloride saline solution. The fractionated samples of rabbit serums A, B, and C were dialyzed against the same concentration of saline solution for 24 hours at 4 C. They were also diluted to a 1:5 dilution of the original serum volume. Photographs were taken every eight minutes after rotor speed reached 60,000 r.p.m. The sedimentation constants were calculated by Svedberg's equation. However,

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* Details of the International Standard Method are described in WHO Tech. Rep. Ser. 67, 1953.

† The Japanese Standard Method is described in "Diagnostic Studies of Contagious Diseases in Domestic Animals," Buneido, Tokyo, 1953.

the measurements of the distance from margin to the peaks were made following the enlargement of the films in a projection room. Each constant was corrected in the usual manner to give $S_{20°,w}$. This assumed that the temperature of the rotor during a run is the average of the readings taken before and after the run. (Refrigeration was not used.)

Electrophoresis.—The electrophoretic studies were carried out at 15 C. in a Tiselius electrophoresis apparatus.* Phosphate buffer solution at pH 8.0, ionic strength 0.144, was used for 40-minute runs in the apparatus. Each serum and the fractionated sample were dialyzed for 24 hours against the same buffer solution (at 4 C.). After dialysis, each sample was diluted to 1:3 of the original serum volume with the same buffer. As far as possible, the same electrical conditions, time, and bar angles were kept when each photograph was taken.

Paper Electrophoresis.—The paper electrophoresis apparatus used was the horizontal Wieland-Fisher type.** Veronal buffer, at pH 8.6, ionic strength 0.10, was used in all instances. Four filter-paper strips (2.5 by 50 cm.) were stretched in a frame made of plastic. (The plastic weights were attached to each end with the ends hanging into beakers containing the electrolyte and electrodes.) At about the middle of the strip, a drop of serum sample was placed on the paper with a glass capillary tube. Care was taken to avoid touching the two sides of the filter paper. A constant current was maintained electronically; however, there was some change in voltage and temperature in the chamber during the run. The average voltage and amperage during four-hour runs was 340 v. and 7 ma. for the four strips.

Agglutination Test on Filter Paper.—For the test, a stained Brucella ring-test antigen ‡ was applied in all instances. Before attempting a new technique of electrophoretic analysis, all materials were tested, using the Hess and Roepke method [8] and the Spalding and Metcalf method [14] with the following minor modification: Normal serum was used for serial dilution and control instead of saline solution, since this gave better results. An attempt was made to apply paper electrophoresis to the previous fixation test method. At the end of the run, each strip was immediately cut lengthwise into two pieces. One piece was used for the determination of the position to which components had migrated by staining in aqueous bromphenol blue; the other strip, while still moist, was used for the fixation test in the following manner (fig. 3): First, the stained Brucella antigen was placed along a vertical line, about 7 mm. from the edge, with a glass capillary tube. Then the strip was looped and irrigated for a few minutes in a large pewter dish containing about 2 ml. of veronal buffer which was used for paper electrophoresis. This caused the chromatographic development of the stained Brucella antigen at right angles to the drawn line only where there was no antibody. The strips were dried at room temperature.

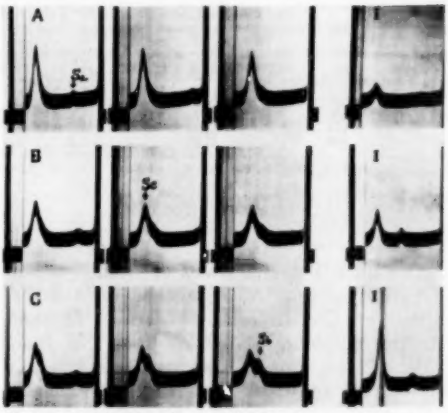

Fig. 1—Sedimentation patterns of serums A (normal serum), B, and C (hyperimmune antiserums), and fraction I of each. Bar angle, 60 degrees; photographs at eight-minute intervals; average speed, 60,800 r.p.m.

RESULTS

Twenty bovine serums, obtained from cattle which had been maintained in quarantine as brucellosis-suspicious, were tested for nonspecific agglutinating substances by the Hess and Roepke method [8] and the new technique described in this paper. The results were negative.

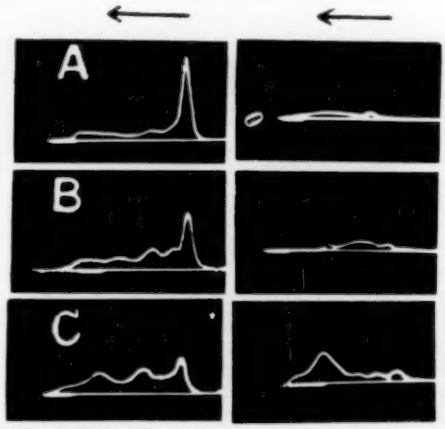

Fig. 2—Descending electrophoretic patterns of rabbit serums A, B, C and fraction I of each. Electrophoretic time: 2,400 seconds.

* Constructed by the Hitachi Co. in Japan.

** Made by the Toyo Scientific Co. in Japan.

‡ Produced by the National Institute of Animal Health, Tokyo, Japan.

TABLE 1—Antibody Titers of Fractions of Serums B and C

Serum	Fraction	Serum dilution											
		1:50	1:100	1:400	1:600	1:800	1:1200	1:1600	1:2000	1:3200	1:4000	1:6400	1:8000
B	I	3+	3+	3+	3+	3+	3+	3+	1+	—	—	—	—
	II	3+	3+	3+	2+	—	—	—	—	—	—	—	—
	III	±	—	—	—	—	—	—	—	—	—	—	—
	IV	—	—	—	—	—	—	—	—	—	—	—	—
C	I	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	1+	—
	II	3+	3+	3+	3+	3+	1+	—	—	—	—	—	—
	III	1+	—	—	—	—	—	—	—	—	—	—	—
	IV	—	—	—	—	—	—	—	—	—	—	—	—

The anti-Brucella titers of each fraction of rabbit serums A, B, and C were determined by the Japanese Standard Method for the tube-agglutination test. No fraction of the normal serum (serum A) showed an agglutination reaction. The results of the titration of serums B and C are shown (table 1).

Ultracentrifugal Analysis.—Two sedimenting components (peaks Sa and Sc) have been observed in serum A, as shown (fig. 1). The first small peak, Sa, which is observed in all cases, would be formed by certain conjugated proteins such as lipoprotein. Another main component of serum A is shown as peak Sc, which is considered the albumin component. In serums B and C, another component (peak Sb) was observed between the two peaks, Sa and Sc. This component was greater in serum C, paralleling the increase in the titers. It is thought to be the globulin component.[10] On the contrary, the albumin components in serums B and C gradually decreased. The sedimentation constants of the main components of each serum and fraction are shown (table 2).

TABLE 2—Sedimentation Constants ($S_{20°}$, ω,) of the Components of Serums A, B, and C and Their Fraction I

Peak	Whole serum			Fraction I
	Sa	Sb	Sc	Sb
Serum A	16.7	....	3.8	6.4
Serum B	16.5	6.4	3.9	6.2
Serum C	16.4	6.1	3.6	6.0

Electrophoresis.—In parallel with ultracentrifugal analysis, electrophoretic analysis was carried out on the same materials. Photographs (fig. 2) show distinct differences in serum components of serums A, B, and C. Globulin components, especially *gamma*-globulin component, of serums B and C increased along with the increase in the titers. The peaks of albumin were markedly lowered. Referring to the photographs of fraction I, a low and flat peak has been observed in serum A, as compared with the peak of *gamma*-globulin of serum C.

Paper Electrophoresis.—The same materials as in two preceding experiments were applied in this experiment. The upper half of each strip, which was stained with aqueous bromphenol blue after the run, shows the distribution of the serum protein components (fig. 3). The pattern of the distribution of serum components is similar to that of the Durrum method.[5] Nearly the same changes in the amount of serum protein components have been observed. The other half of each strip shows the positions of the agglutinating substances which were made visible by the fixation of stained cells. In this experiment with agglutinins present, stained cells were fixed and remained on the original line. Where there is no such substance, the cells may ascend freely in the paper to the top edge of the strip.

Figure 3 illustrates the positions and the distribution of the components of agglutinating substances. Although no reaction has been observed in the materials of serum A, the results with serums B and C illustrate the presence of the agglutinins on and between *gamma*-globulin and *beta*-globulin areas. The reaction was observed around the starting line in the tests of fraction I of serums B and C.

DISCUSSION

The value of paper electrophoresis of bovine serum has been reported recently.[13] When immune serum is fractionated by means of salting out, using four concentrations of ammonium sulfate, most antibodies are found in the first, or *gamma*-globulin, fraction.[15] It is possible to separate the antibodies into several fractions by ethanol precipitation.[9] However, antibodies may occur in more than one serum fraction.

This is shown by the work of Enders [6] and Cann *et al.*,[1,3] who used an electrophoresis convection method, and also by Payne and Deming,[12] who studied the electrophoretic mobility in paper of isoagglutinins. In the present experiment, fraction II, the so-called *beta*-fraction, also had fairly high titers. This illustrates that agglutinating substances move not only with the *gamma*-globulin fraction but also with the *beta*-globulin fraction. This was verified by the fixation test on filter paper with paper electrophoresis.

The relation between ultracentrifugal patterns and serum protein components of each fraction has been discussed in previous reports.[2,10] According to those experiments, usually three boundaries were observed in the ultracentrifugal analysis of normal serum. The first peak which has a large sedimentation constant, usually around 17s, is considered as the peak of a certain conjugated protein component. This component was present not only in fraction I, but in fractions II and III. However, there were no specific changes observed in the amount of this component throughout the course of the immunization. Therefore, it is not considered that this component participates directly in agglutination reactions.

The second peak, which has a lower sedimentation velocity, is the peak of globulin components. The peak was not seen in rabbit serums before immunization. After immunization, however, the peak began to appear in parallel with the rise in titer. The same results were seen in the analysis of each fraction.

The third peak, which is the main component of fraction IV, is the peak of the albumin component. This is not considered the direct carrier of the agglutinating substances, yet the amount of this component was gradually decreased during the course of immunization. The same relationship, in all respects, has been demonstrated from the electrophoretic analysis of the same materials. Although further investigation should be made, as far as Brucella is concerned, the homologous results have been obtained from the analysis both by using ultracentrifugal and electrophoretic methods. However, in previous experiments,[11] there were some cases which yielded different results from those shown in the electrophoretic pictures, *i.e.*, globulin increased whereas albumin decreased, but ultracentrifugal patterns showed reverse results.

The Hess and Roepke method was used with all rabbit and bovine serums to investigate whether nonspecific Brucella agglutinating substances were present. However, we obtained no results similar to those

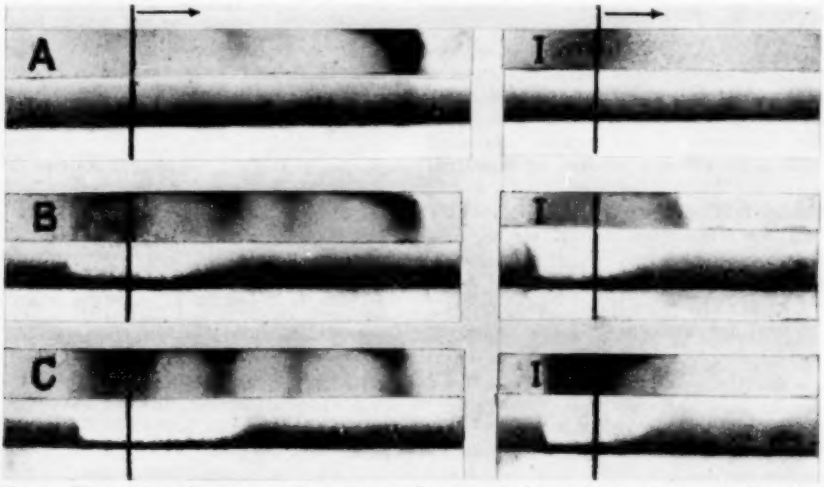

Fig. 3—Electrophoretic paper strip patterns of serum A (normal serum), serums B and C (hyperimmune serums), and fraction I of each. The upper half of each strip illustrates the location of serum components and the lower half the chromatographic development and location of stained Brucella antigen.

described in their paper.[8] The fixation reaction occurred in all cases on the starting spots where they said a nonspecific reaction might take place. No fixation was found at any other place. This means that all agglutinative substances, including specific agglutinating substances, remained stationary on the original spots in this experiment.

It is difficult to suggest reasons why these opposite results occurred in our experiment. It might be because of the difference in the quality of filter paper or, possibly, other minor technical differences. Therefore, in order to resolve the discrepancy, paper electrophoresis was applied in an attempt to separate the agglutinative substances on the filter paper instead of using the method of simple chromatography. However, the agglutination reaction occurred in a wide range on and between the *gamma-* and *beta*-globulin areas, and there was no fixation observed in other places. It is, of course, impossible to distinguish a nonspecific Brucella agglutinative substance from specific substances if it moves with other specific agglutinative substances in the electrophoretic field. If not, other results might be expected. Therefore, further experiments are necessary to determine the presence or absence of a nonspecific substance in the materials used in the present experiment.

SUMMARY

The titration of *gamma-*, *beta-*, and *alpha*-globulin and albumin fractions of Brucella hyperimmune rabbit antiserums was carried out. The titers of *gamma-* and *beta*-globulin fractions of the serums increased proportionally after a course of immunization.

Ultracentrifugal and electrophoretic analyses were made on rabbit serums obtained before and after a course of immunization and on bovine serums obtained from cattle which were considered "suspicious" when tested for brucellosis. The same results were observed in both analytical methods.

The fixation test following paper electrophoresis was made on immunized rabbit and bovine serums. The reaction occurred in an area between the *gamma*-globulin fraction and the *beta*-globulin fraction.

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A Rapid Macroscopic-Slide Screening Test for the Serodiagnosis of Leptospirosis

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FEW public health, medical, and veterinary diagnostic laboratories offer a service for the serodiagnosis of leptospirosis by the microscopic-agglutination test due, primarily, to the tedious and time-consuming techniques involved and the instability of the leptospiral antigens.

Rapid slide-agglutination tests for the enteric fevers (typhoid and paratyphoid),[1,11,12] brucellosis,[4] and tularemia[6] are well established and have been used satisfactorily for many years. The need for similar rapid tests for leptospirosis has led to the development of several[3,7,8] leptospiral macroscopic slide-agglutinating antigens. Of these, only the Stoenner rapid plate antigen[8] has proved satisfactory. The method of preparation of this antigen requires multiple periods of centrifugation for a total of three hours and 15 minutes. It was felt that a simpler technique would be more practical for use in the average diagnostic laboratory.

In an effort to develop satisfactory, stable leptospiral antigens which could be prepared with a minimum amount of manipulation, certain phases of the techniques used[1,4,6,8,12] in other macroscopic-slide tests were combined and modified. This paper describes the method which resulted in the preparation of leptospiral rapid slide-agglutination test antigens and the technique of the test. An evaluation of the sensitivity of the antigens after storage periods up to 18 months is presented. The report includes data accumulated during the comparative use of the rapid slide test and the microscopic-agglutination test with human, bovine, canine, and porcine serums over a two-year period. These comparative studies indicate that the macroscopic-slide test may be used satisfactorily as a screening test on human and animal serums.

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MATERIALS AND METHODS

The leptospiral strains used in the preparation of the rapid slide test antigens included the following nine cultures: *Leptospira autumnalis* AB, Akiyama A; *Leptospira ballum*, S102; *Leptospira bataviae*, Van Tienen; *Leptospira canicola*, Ruebush; *Leptospira grippotyphosa*, Andaman; *Leptospira icterohaemorrhagiae* AB, Wijnberg; *Leptospira pomona*, S-91; *Leptospira pyrogenes*, Salinem; and *Leptospira sejroe*, Mallersdorf. These strains were used also in the preparation of the microscopic-agglutination test antigens. The procedure used to prepare the microscopic test antigens and to perform the test was described in an earlier report.[2]

Preparation of Antigens.—The antigens for the test were prepared as follows:

1) The leptospiras were grown in Stuart's medium.[9] The medium was dispensed in 500-ml. amounts in 32-oz. screw-capped prescription bottles and inoculated with 50 ml. (1:10 ratio) of an actively growing 4- to 6-day-old leptospiral culture per bottle. The inoculated bottles were incubated in a slanted position at 28 to 30 C. for four to six days. After incubation, the cultures were examined by dark-ground microscopy for density, autoagglutination, and contamination. If satisfactory, formalin was added to a final concentration of 0.5 per cent and the cultures were allowed to stand for one to two hours. If necessary, the formolized cultures may stand for 24 hours.

2) The killed antigens were then centrifuged in 50-ml. tubes for ten minutes at 1,500 g to remove extraneous material. They were checked again by dark-ground microscopy for autoagglutination, and only smooth nonclumping cultures were used.

3) The supernatant fluid was transferred to 50-ml. (plastic) tubes and centrifuged in an SS-1 Servall angle head centrifuge for 25 minutes at 5,000 g (or 15 min. at 9,000 g) to pack the leptospiral cells.

4) The supernatant fluid was carefully poured off immediately and the tubes were allowed to drain in a slanted position.

5) The sedimented cells were resuspended in 1.5 to 2.0 ml. (per tube) of a solution containing 0.5 per cent formalin, 12.0 per cent sodium chloride, and 20.0 per cent glycerine (cells from 40 ml. of culture). The antigen was mixed 15 to 20 times using a 2-ml. Cornwall pipette with a No.

16- to 18-gauge needle, and pooled in 6- to 8-oz. prescription bottles for further processing. If the cells failed to resuspend completely, as determined by dark-ground microscopy, the clumps were removed by centrifugation for ten minutes at 1,500 g. Thus, 1 liter of culture was concentrated to 50 to 60 ml. of antigen. The final yield of antigen per liter of culture after standardization varied from 5 to 10 per cent.

6) The antigens were refrigerated at 4 C. for two to four days before standardization.

7) In standardization of the antigens for density, factors of prime consideration were to obtain an antigen that could be read with ease macroscopically and, at the same time, would compare in sensitivity to the microscopic-agglutination test. Varying densities of antigen, determined by measuring the percentage of light transmission from 20 to 80 per cent on a Lumetron photoelectric colorimeter were tested both for sensitivity and ease of reading. Bovine, canine, or human serum samples of known titer and which were negative by the microscopic-agglutination test (with formolized antigens), and hyperimmune rabbit serums were used to check sensitivity. Serums with titers ranging from 1:16 to 1:8,192 were included. It was found that antigens adjusted to 25 to 26 per cent light transmission, by the Lumetron photoelectric colorimeter with a No. M550 filter and No. 6 reduction plate, were not only read with less difficulty but compared favorably in sensitivity with the microscopic-agglutination test. The pH of the completed antigens ranged from 5.4 to 5.8. The antigens were stored at 4 C.

It was observed earlier, while testing the sensitivity of varying densities of microscopic-agglutination test antigen, that prozone reactions increased as the density of antigen decreased. Thus, it was considered that high titer serums which exhibited zones of inhibition in the lower dilutions may show a weak reaction or be missed entirely with a dilute antigen when screening with the undiluted serum. To test this possibility, eight bovine serums which had shown prozones in the microscopic-agglutination test up to dilutions of 1:128 to 1:512 to *L. pomona* antigen were selected for examination with *L. pomona* slide antigens adjusted to 25, 50, and 75 per cent light transmission. It was found that, with one exception, all reactions in the screening dilution (0.01 ml. undiluted serum plus 0.055 ml. antigen) were weaker with antigens adjusted to 50 per cent light transmission than with antigens adjusted to 25 per cent light transmission; at 75 per cent one specimen showed no reaction (table 1).

Equipment for the Test.—The following equipment is necessary to perform the test: (1) large glass plate, 8 by 15 inches, etched in 1-inch squares, as used for Brucella testing, or any similar type of clear glass plate marked in 1-inch squares; (2) 0.2-ml. pipettes marked in 0.001 ml.; (3) applicators or toothpicks; (4) mechanical slide rotator with speed regulator; (5) a good indirect light source with black background (the Minnesota Testing Box has been found convenient); (6) antigen-dropping bottles * (30-ml. brown glass bottles). The bulblike tip of the dropper pipette has been found to deliver drops of comparable amount (about 0.055 ml.).

Procedure for Performing the Test.—The rapid slide-agglutination test should be performed as follows:

1) For each antigen used, place 0.01 ml. of the serum to be tested on the glass plate with a 0.2-ml. pipette. Add 1 drop of the desired antigen with the dropper pipette.

2) Mix serum and antigen thoroughly with applicator stick or toothpick and rotate plate by hand five to ten times.

3) Place plate on mechanical rotator, with speed regulator set for 125 r.p.m., for four minutes.

4) Remove and read reaction over light box. The clumps of agglutinating leptospiras occasionally tend to gather around the periphery of the drop, but will be more evenly distributed by gentle

* Bottles (No. 18060) obtained from A. S. Aloe Co., St. Louis, Mo.

TABLE 1—Comparison of Eight Bovine Serums Exhibiting Prozone Reactions by the Microscopic-Agglutination Test with Varying Densities of Antigen by the Slide Test

Bovine serum (No.)	Microscopic-agglutination titer with *L. pomona* antigen													Slide agglutination with *L. Pomona* antigen		
	1*	2	3	4	5	6	7	8	9	10	11	12	13	25% LT† #1	50% LT #1	75% LT #1
2661	±	—	—	—	—	—	1	4	4	3	2	1	—	4	4	3
2669	±	—	—	—	±	3	4	4	3	2	—	—	—	4	2	2
2673	±	±	—	—	1	4	4	3	2	—	—	—	—	4	2	2
2674	—	—	—	—	3	4	3	2	—	—	—	—	—	4	3	2
2677	1	1	±	—	—	2	4	4	4	1	—	—	—	4	1	1
2727	1	—	—	—	1	3	4	4	3	1	—	—	—	4	2	2
2729	1	1	1	1	4	4	4	3	1	—	—	—	—	4	3	2
2731	2	2	1	—	—	2	3	2	1	—	—	—	—	3	2	—

* Numbers represent twofold serum dilutions beginning at 1:16; † percentage of light transmission.

Degree of agglutination is indicated as 1, 2, 3, or 4; ± = trace of agglutination or doubtful reaction; — = no agglutination; #1 = code number representing screening dilution of serum (0.01 ml. undiluted serum + 1 drop of antigen).

hand rotation of the plate. Degree of agglutination is recorded as 4+ when all organisms appear clumped and the supernatant fluid is clear, 3+ if approximately 75 per cent of the leptospiras have agglutinated, 2+ with 50 per cent agglutination, and 1+ with 25 per cent agglutination. No agglutination characterized by an even suspension of serum-antigen mixture is recorded as negative.

5) If the positive reaction is obtained in one or more antigens, the serum is diluted 1:5 and titrated against those antigens.

6) For titrations, the diluted serum (1:5) is placed on the glass plate in 0.04-, 0.02-, 0.01-, and 0.005-ml. amounts. A drop of the desired antigen is placed on each drop of diluted serum, mixed, rotated, and read as above.

For convenience in recording end points, the serum dilutions are coded in ascending order as 1 (negative) and 1, 2, 3, 4, and 5 (positive) in all tabular material.

At first, serums were screened undiluted as in step 1, with six single antigens *(L. icterohaemorrhagiae, L. canicola, L. ballum, L. autumnalis, L. pomona,* and *L. sejroe).* Later, when antigens of *L. bataviae, L. grippotyphosa,* and *L. pyrogenes* were added to the battery, three pools of the nine antigens were prepared. Equal portions of three antigens were mixed for the following pools: pool 1—*L. icterohaemorrhagiae, L. canicola,* and *L. ballum;* pool 2—*L. bataviae, L. grippotyphosa,* and *L. pyrogenes;* and pool 3—*L. autumnalis, L. pomona,* and *L. sejroe.*

When cultures isolated from wild animals in southwestern Georgia proved to be members of the *australis A, hyos,* and *hebdomadis* sero-groups,[2,9] antigens were prepared from these strains. For screening, these antigens are combined to form pool 4. The fourth pool, which was prepared only recently, was not included in the results reported here.

For screening, 1 drop of each of the pooled antigens is added to 0.01-ml. amounts of undiluted serum, mixed, and rotated as in steps 2 and 3. Results of reactions are recorded as positive (+) where distinct clumping occurs, doubtful (±) if only slight agglutination is noticed, and negative. All that show positive and doubtful reactions are then tested with the single antigens comprising the respective pool.

The serum samples used in this study for comparative examination by the slide- and microscopic-agglutination tests were collected primarily by members of the laboratory staff during field investigations of suspected cases of leptospirosis in human beings, dogs, or cattle. Other bovine, canine, and porcine samples were submitted to the laboratory by practicing veterinarians, and additional human serum samples were obtained from the Communicable Disease Center Diagnostic Serology Unit, where all reference specimens from the states are examined. Serial bovine samples (98) from 9 calves experimentally infected with *L. sejroe,* 7368, also were studied comparatively. Many of the bovine samples were obtained from dairy or beef herds during epizootics of leptospirosis.

As the highest percentage of bovine and por-

TABLE 2—Findings on Retesting of Human, Canine, and Bovine Serums with Different Lots of Six Leptospiral Macroscopic-Slide Test Antigens of Varying Age to Demonstrate Stability of the Antigens

Antigen		Serum dilutions							
		Negative	Positive					Total examined	Range of * difference not over
Serotype and lot No.	Age (months)	1	1	2	3	4	5		
L. autumnalis V	1.5	10	2	0	3	2	5	22	2
L. autumnalis III	5	10	1	1	4	3	3		
L. autumnalis I	9	10	0	1	1	2	8		
L. ballum III	2	11	2	3	3	2	2	23	2
L. ballum II	4	11	2	3	4	2	1		
L. ballum I	9.5	12	3	1	3	3	1		
L. canicola VIII	17 days	9	5	1	0	2	5	22	1
L. canicola VII	3.5	9	5	1	1	3	3		
L. canicola VI	9	9	4	2	0	2	5		
L. canicola IV	14	9	6	0	2	2	3		
L. canicola III	17	9	5	1	0	2	5		
L. ictero.† X	1	11	3	2	1	4	4	25	2
L. ictero. IX	4.5	11	0	3	1	4	6		
L. ictero. VI	9.5	10	1	1	3	3	7		
L. ictero. III	16	9	1	2	3	3	7		
L. pomona XII	1	7	5	1	5	2	5	25	2
L. pomona XI	6	7	4	2	2	4	6		
L. pomona IX	9	7	3	3	6	1	5		
L. sejroe XIII	1	10	1	1	3	3	3	21	2
L. sejroe XII	1.5	10	1	2	3	1	4		
L. sejroe XI	2.5	11	0	2	2	2	4		
L. sejroe IX	6	10	1	0	4	1	5		
L. sejroe V	13	11	2	2	1	1	4		
L. sejroe II	18	10	1	3	1	2	4		

* The range of difference in reaction of any serum with different lots of the six antigens was not more than one or two dilutions, as indicated; † *L. icterohaemorrhagiae.*

cine serums showed reactions with *L. pomona* antigen, results of slide and microscopic-agglutination tests with the respective antigens of this serotype were selected for comparison, except in the series of samples from *L. sejroe*-infected calves. Similarly, the results with *L. canicola* antigens by the two tests with canine serums were compared.

RESULTS

Stability of Slide Test Antigens.—Certain lots of the slide antigens were held for one to 18 months and then examined for stability and sensitivity. Included in this study were several lots each of antigens prepared from six serotypes, including *L. autumnalis*, *L. ballum*, *L. canicola*, *L. icterohaemorrhagiae*, *L. pomona*, and *L. sejroe*. Serums used in the evaluation of antigens for the first four serotypes were obtained from suspected or proved cases in human beings or dogs, or both, or from normal individuals. The microscopic-agglutination and slide tests had been performed on these serums when they were obtained, and they were then frozen for one to 12 months. The microscopic titer was rechecked the same day the slide antigens were retested. Serums from cattle which had shown microscopic-agglutination titers to *L. pomona* and serums from normal cattle were used to test the *L. pomona* antigens. Serums of varying microscopic titers to *L. sejroe* from calves experimentally infected with *L. sejroe* and normal cattle serums were used for antigens of this serotype.

To test the stability of *L. canicola*, *L. icterohaemorrhagiae*, *L. autumnalis*, and *L. ballum* antigens, both canine and human serums of known titer to the respective serotypes by the microscopic-agglutination test and negative serums were used. Of the positive serums in this series in which infection was proved by isolation, only *L. canicola* and *L. icterohaemorrhagiae* were obtained. The 24 antigens of six serotypes ranged from 17 days to 18 months old. The findings of the slide tests (table 2) indicate very little variation within the lots of antigen prepared from the same serotype and tested at different ages with the same serums.

The range of difference in titer in any two serums with the respective antigens was found to be no greater than two dilutions. These differences occurred more commonly between the second and fourth dilutions. One lot of *L. pomona* antigen was too sensitive when tested at 14 months of age and was not included in this series. However, it compared favorably with the microscopic test when used at 12 months.

Comparative Examination of Domestic Animal Serums by Slide- and Microscopic-Agglutination Tests.—For several months after the slide test antigens were developed, a mechanical rotator was not available. During this period, a series of 575 bovine serum samples was tested comparatively by the slide- and microscopic-agglutination tests with the respective *L. pomona* antigens. Slide tests were rotated by hand five to six times, allowed to stand in the covered testing box for six minutes,

TABLE 3—Comparative Findings on 575 Bovine Serums by the Slide-Agglutination Test, Performed Without Rotator, and the Microscopic-Agglutination Test with the Respective Leptospira Pomona Antigens

Microscopic titer	Slide titer						
	Negative	Positive					
	1	1	2	3	4	5	Total
Negative (1:16)	288	14	3	....	....	....	305
Positive (1:16)	19	3	4	....	....	....	26
1:32	15	4	11	....	....	....	30
1:64	8	5	14	13	1	....	41
1:128	....	2	12	7	6	....	27
1:256	....	....	3	14	21	8	46
1:512	....	....	1	3	8	26	38
1:1,024	....	....	....	....	7	33	40
1:2,048	....	....	....	....	....	12	12
1:4,096	....	....	....	....	....	9	9
1:8,192	....	....	....	....	....	1	1
Total	330	28	48	37	43	89	575

Correlation coefficient = 0.88.

TABLE 4—Comparative Findings on 576 Bovine Serum Samples by the Slide- and Microscopic-Agglutination Tests Performed with Rotator and the Respective Leptospira Pomona Antigens

Microscopic titer	Slide titer						
	Negative	Positive					
	1	1	2	3	4	5	Total
Negative (1:16)	110	35	9	....	....	....	154
Positive (1:16)	5	33	24	7	....	....	69
1:32	2	11	28	17	2	1	61
1:64	....	8	14	20	11	6	59
1:128	....	2	9	11	18	12	52
1:256	....	....	3	4	9	21	37
1:512	....	....	....	....	2	41	43
1:1,024	....	....	....	....	1	36	37
1:2,048	....	....	....	....	....	24	24
1:4,096	....	....	....	....	....	18	18
1:8,192	....	....	....	....	....	11	11
1:16,384	....	....	....	....	....	8	8
1:32,768	....	....	....	....	....	3	3
Total	117	89	87	59	43	181	576

Correlation coefficient = 0.82.

then rotated another 15 times as recommended by Stoenner.[8] By this method, the slide test appeared somewhat less sensitive than the microscopic test, with 42 serums appearing negative that showed low titers of 1:16 to 1:64 (table 3). The mechanical rotator was used for all subsequent bovine samples.

Comparative slide and microscopic findings with *L. pomona* antigens on 576 bovine serums performed with the rotator are shown (table 4). A total of 459 were positive by the slide test, as compared with 422 by the microscopic-agglutination test. Only seven serums showed microscopic titers of 1:32 or less that were missed by the slide test.

An opportunity to test the sensitivity of *L. sejroe* slide antigen with bovine serum was provided by 98 samples from 9 calves experimentally infected with *L. sejroe*. There were 76 samples positive and 19 negative by both tests, while the remaining three were positive by the slide test and negative by microscopic-agglutination test (table 5). These three samples were collected on the fourth, fourth, and sixth days postinoculation, respectively. Samples collected 48 hours later from these 3 animals were positive by both tests. These 98 samples were negative by slide test with *L. autumnalis, L. ballum, L. bataviae, L. canicola, L. grippotyphosa, L. icterohaemorrhagiae, L. pomona,* and *L. pyrogenes* antigens.

TABLE 5—Comparative Findings by Slide- and Microscopic-Agglutination Tests on 98 Serial Bovine Serum Samples from 9 Calves Experimentally Infected with Leptospira Sejroe Antigens

Microscopic titer	Slide titer: Negative 1	Positive 1	2	3	4	5	Total
Negative (1:16)	19	3	....	....	....	....	22
Positive (1:16)	....	....	1	....	....	....	1
1:32	....	....	3	2	....	....	5
1:64	....	....	....	3	5	....	8
1:128	....	....	....	3	4	3	10
1:256	....	....	1	....	3	10	14
1:512	....	....	....	....	....	11	11
1:1,024	....	....	....	....	....	13	13
1:2,048	....	....	....	....	....	7	7
1:4,096	....	....	....	....	....	3	3
1:8,102	....	....	....	....	....	2	2
1:16,384	....	....	....	....	....	2	2
Total	19	3	5	8	12	51	98

Correlation coefficient = 0.93.

A total of 265 canine serum samples were compared by both tests with *L. canicola* antigens. Of these, 158 (60%) were positive. There were no samples that were positive in one test and negative in the other (table 6).

TABLE 6—Comparative Findings on 265 Canine Serums by the Slide- and Microscopic-Agglutination Tests with Leptospira Canicola Antigens

Microscopic titer	Slide titer: Negative 1	Positive 1	2	3	4	5	Total
Negative (1:16)	107	....	....	....	....	....	107
Positive (1:16)	....	2	....	....	....	....	2
1:32	....	3	1	....	....	....	4
1:64	....	7	5	5	....	....	17
1:128	....	3	4	7	2	....	16
1:256	....	....	4	7	10	6	27
1:512	....	....	....	....	6	22	28
1:1,024	....	....	....	....	3	10	13
1:2,048	....	....	....	....	....	18	18
1:4,096	....	....	....	....	....	19	19
1:8,192	....	....	....	....	....	8	8
1:16,384	....	....	....	....	....	5	5
1:32,768	....	....	....	....	....	1	1
Total	107	15	14	19	21	89	265

Correlation coefficient = 0.97.

Comparative tests on 81 porcine serums with *L. pomona* antigens yielded 73 positive by the slide test and 71 positive by the microscopic-agglutination test (table 7).

For each of the five sets of data in which titers obtained by the microscopic-tube and slide tests were compared, correlation coefficients were computed to describe the relationship between the tests. The actual dilutions of 1:6.5, 1:11.9, 1:18.8, 1:32.5, and 1:60.0 for the slide test were used. After taking logarithms of these end points and the corresponding microscopic test end points, a midpoint was determined for each interval between logarithms. Thus,

TABLE 7—Comparative Findings on 81 Porcine Serum Samples Examined by Slide- and Microscopic-Agglutination Tests with Leptospira Pomona Antigens

Microscopic titer	Slide titer: Negative 1	Positive 1	2	3	4	5	Total
Negative (1:16)	6	4	....	....	....	....	10
Positive (1:16)	1	7	5	5	1	....	19
1:32	1	....	1	7	1	....	10
1:64	....	....	1	3	4	1	9
1:128	....	....	....	3	....	....	3
1:256	....	....	....	1	5	2	8
1:512	....	....	....	....	5	10	15
1:1,024	....	....	....	....	....	3	3
1:2,048	....	....	....	....	....	4	4
Total	8	11	7	19	16	20	81

Correlation coefficient = 0.77.

for microscopic test titers of 1:32 and 1:64, the logarithms to the base two were five and six, with a midpoint of 5.5. These midpoints were used in the calculations to represent the serums which reacted at the lower dilution used to compute the midpoint but not at the higher dilution. In this example, 5.5 would be used for serums with a microscopic test titer of 1:32.

The correlation coefficients computed from the midpoint logarithms (tables 3-7) were 0.88, 0.82, 0.93, 0.97, and 0.77, respectively. All of these are significantly different from zero (which represents no correlation) at a probability level of 0.001. No comparison among the correlation coefficients is feasible because of the different serums tested.

In order to indicate the approximate slide test titer corresponding to any microscopic test titer, median slide test titers are given (table 8) for each microscopic test titer from each set of data in tables 3 through 7. As mentioned previously, slide test end points were coded in ascending order as negative, 1, 2, 3, 4, and 5. The medians were rounded to the nearest end point lower than the median. Thus, a median of 4.7 was recorded as 4.

It may be noted that the slide test was not as sensitive as the microscopic-tube test when performed on bovine serums without use of a mechanical rotator (data from table 3). However, this was not evident with bovine serums when a mechanical rotator was used (data from table 4). In general, the slide test median titer increased gradually with the microscopic test titer up to the final slide test dilution indicated as five. For each microscopic test titer of 1:512 or above, the slide test median titer was five. Thus, by slide test titration, it is possible to predict the approximate microscopic test titer from 1:16 to 1:512.

Screening with Pooled Antigens.—To determine whether the slide antigens were sufficiently sensitive for screening when equal amounts of three types were pooled, serums were also tested by microscopic agglutination with *L. ballum, L. canicola, L. icterohaemorrhagiae, L. autumnalis, L. pomona,* and *L. sejroe* antigens. If positive by the microscopic test and negative by slide test with pooled antigens, the single slide antigens were then checked.

Bovine serum samples were frequently obtained in large groups from single herds. If preliminary testing indicated that the titers were predominantly to *L. pomona,* the entire herd was not screened with the pooled antigens but with single antigens of pool 3. However, a total of 570 bovine samples were screened with pooled antigens and also checked by microscopic agglutination. Of these, 329 were positive in one or more pooled antigens, 33 showed a plus-minus (doubtful) reaction and 208 were negative. By the microscopic test, 320 were positive in dilutions of 1:16 to 1:32,798 with one or more antigens, and 250 were negative. There were only ten samples which were negative by pooled antigens that showed a titer by microscopic agglutination. These include five at 1:16, four at 1:32, and one at 1:64, titers which are considered of little significance. There were 31 samples that showed a positive reaction in pooled antigens and 18 that showed a doubtful reaction that were negative by the microscopic test.

The first 205 canine serums were tested only with pool 1, but pools 1, 2, and 3 were used on the last 60 samples. A total of 169 samples were positive in one or more of the pools. All of these reacted with single antigens by slide and microscopic tests. Only one of the 96 samples that were negative in pooled antigens showed a positive reaction in the other

TABLE 8—Median Macroscopic-Slide Test Titer for Each Microscopic Test Titer *

Data used from table No.	Microscopic-agglutination test titer						
	Negative	1:16	1:32	1:64	1:128	1:256	1:512
3	Neg.	Neg.	Neg.	2	3	4	5
4	Neg.	1	2	3	4	5	5
5	Neg.	2	2	4	4	5	5
6	Neg.	1	1	2	3	4	5
7	Neg.	2	3	4	3	4	5

* The slide-agglutination test median titer was 5 for each microscopic-agglutination test titer of 1:512 or greater.

tests, and that gave a weak reaction with undiluted serum in *L. canicola*, slide antigen, and a 1:16 *L. canicola* titer by microscopic test. One canine sample showed a 1:512 titer by microscopic agglutination with *L. autumnalis* antigen. This sample was positive only in pool 3, which contained *L. autumnalis* antigen.

Pools 1, 2, and 3 were used on 114 of the human samples but 1 and 3 only were used with 57 samples. Of the 171 human serums, 75 showed a positive slide-agglutination reaction in one or more of the pooled antigens, six gave a doubtful reaction, and 90 were negative. By the microscopic-agglutination test, 76 reacted with titers ranging from 1:16 to 1:8,192, with one or more of the leptospiral antigens, and 95 were negative. One of the samples that showed a titer of 1:64 to *L. sejroe* antigen gave a doubtful reaction by the slide test with pooled antigens. The remaining five samples that appeared doubtful with pooled slide antigens were negative by microscopic agglutination in a 1:16 dilution.

DISCUSSION

The leptospiral macroscopic slide-agglutinating antigens described are easy to prepare, stable for at least nine to 12 months, and compare favorably in sensitivity to the microscopic-agglutination test. Because of the simplicity of preparation of the antigens and their stability, they provide a method by which small laboratories may easily use a battery of antigens. Until a simple and practical genus-specific test is found, the use of multiple antigens becomes increasingly important. This fact has been re-emphasized recently by the isolation and identification of leptospiral serotypes, hitherto unrecognized in the United States, from wild mammal hosts in southwest Georgia.[2,5]

For screening with large numbers of serum samples, the test was further simplified by combining the 12 antigens into four pools of three antigens each. The pooled antigens were found to be satisfactory for screening human, canine, and bovine serums. Studies in progress indicate they may be used for porcine serums and serums from wild mammals. Thus, for a negative specimen, only four slide tests (0.01 ml. of serum and 1 drop of antigen per test) are necessary. Titration of positive serums is carried out by preparing only one dilution of the serum and using varying amounts of this diluted serum to obtain further dilutions similar to the method used in large-scale Brucella testing. The addition of glycerine to the slide antigens prevents rapid drying of the tests. Glycerine has been found also to provide a protective effect on the stability of bacterial,[1] viral, and rickettsial antigens.[10]

Analysis of comparative data indicates also that the approximate microscopic titer usually may be estimated from the slide test titer, with certain exceptions. For example, dogs naturally infected with *L. canicola* would show comparable titers by both slide and microscopic tests with *L. canicola*, *L. icterohaemorrhagiae*, and *L. ballum* antigens, but with *L. autumnalis* and *L. pomona* antigens this would not always hold true.

Cross reactions with the macroscopic-slide test antigens occurred primarily with acute phase serums. For example, a dog infected with *L. canicola* occasionally showed an equal or higher titer with *L. ballum*, *L. icterohaemorrhagiae*, or *L. pyrogenes* antigens than with *L. canicola* during this stage of illness, and cattle infected with *L. pomona* often showed a stronger reaction with *L. autumnalis* antigen. However, the degree of cross reactivity appeared much lower or was absent entirely in samples from recovered cases and carrier animals. This was clearly demonstrated in the examination of a small series of serums from wild mammals that had yielded leptospiras from cultures of their kidneys. When screened with the four pooled antigens the serums from 2 raccoons were positive in pool 2 only. Tested with the single antigens of this pool they were positive with *L. grippotyphosa* antigen. The isolations from these 2 animals proved to be *L. grippotyphosa*.

SUMMARY

A simple method for the preparation of leptospiral macroscopic slide-agglutination test antigens is described. Different lots of antigens prepared from six leptospiral serotypes were found to be stable for nine months or longer with little or no variation in sensitivity. Antigens for a total of 12 leptospiral serotypes are used routinely.

Comparative tests on 138 human serums, 1,249 bovine serums, 265 canine serums, and 80 porcine serums by slide- and microscopic-agglutination tests indicated that

the slide test is as sensitive as the microscopic test.

For rapid screening, the 12 antigens are combined into four pools of three antigens each.

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